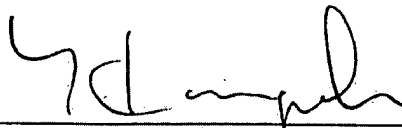## VERIFICATION OF TRANSLATION

Japanese Patent Application No. 10-113962
Filed on April 23, 1998

  I, Yoshiyuki KAWAGUCHI, a citizen of Japan and the translator of the document attached, whose address is c/o SERA, TOYAMA, MATSUKURA & KAWAGUCHI, Acropolis 21 Building, 6$^{th}$ Floor, 4-10, Higashi Nihonbashi 3-chome, Chuo-Ku, Tokyo, Japan, state that the following is a true translation of the Japanese Patent Application No. 10-113962 filed on April 23, 1998 to the best of my knowledge and belief.

Signed at Tokyo, Japan
This 17th day of July, 2008

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[Indication of Official Fee]
      [Ledger Number]    012092
      [Amount]        21000 yen
[List of Document Submitted]
      [Name of Document] Specification    1 copy
      [Name of Document] Drawings     1 copy
      [Name of Document] Abstract     1 copy
      [Number of General Power of Attorney]    9117157
[Necessity of Proof]    Yes

[Name of document] Specification
[Title of invention] Substance with antithrombic
activity and method for detecting glycocalicin

[Claims]
 1. A method for detecting binding of von
Willebrand factor and glycoprotein Ib or inhibition
of the binding, comprising the steps of:
 immobilizing von Willebrand factor in a
reaction vessel in the presence of a substance
inducing the binding of von Willebrand factor and
glycoprotein Ib, and,
 reacting the immobilized von Willebrand factor
with glycoprotein Ib.

 2. The method according to Claim 1, wherein the
substance that induces the binding of von Willebrand
factor and glycoprotein Ib is botrocetin.

 3. A method for detecting binding of von
Willebrand factor and glycoprotein Ib or inhibition
of the binding, comprising the steps of:
 binding a chimeric protein that consists of an
Fc region of immunoglobulin molecule fused at its
amino terminus to a partial protein comprising a von
Willebrand factor binding site of glycoprotein Ibα
chain at its carboxyl terminus or the chimeric
protein labeled with a labeling substance to von
Willebrand factor immobilized in a reaction vessel,
and
 detecting the Fc region of the immunoglobulin
molecule or the labeling substance.

 4. The method according to Claim 3, wherein the
substance that induces the binding of von Willebrand
factor and glycoprotein Ib is botrocetin.

5. The method according to Claim 3, wherein von Willebrand factor is immobilized in the reaction vessel in the presence of a substance that induces the binding of von Willebrand factor and glycoprotein Ib.

6. The method according to Claim 1, wherein glycocalicin is measured by adding a sample containing glycocalicin to the reaction vessel when von Willebrand factor is reacted with glycoprotein Ib, or prior to the reacting, and detecting inhibition of the binding of von Willebrand factor and glycoprotein Ib.

7. The method according to Claim 3, wherein glycocalicin is measured by adding a sample containing glycocalicin to the reaction vessel when the chimeric protein is allowed to bind to von Willebrand factor, or prior to the binding, and detecting inhibition of the binding of von Willebrand factor and the chimeric protein.

8. The method according to Claim 1, wherein a substance that inhibits the binding of von Willebrand factor and glycoprotein Ib is detected by adding a sample containing a substance to be detected to the reaction vessel when von Willebrand factor is reacted with glycoprotein Ib, or prior to the reacting, and detecting inhibition of the binding of von Willebrand factor and glycoprotein Ib.

9. The method according to Claim 3, wherein a substance that inhibits the binding of von Willebrand factor and glycoprotein Ib is detected by adding a sample containing a substance to be

detected to the reaction vessel when the chimeric protein is allowed to bind to von Willebrand factor, or prior to the binding, and detecting inhibition of the binding of von Willebrand factor and the chimeric protein.

10. A chimeric protein, which consists of an Fc region of immunoglobulin molecule fused at its amino terminus to a partial protein comprising a von Willebrand factor binding site of glycoprotein Ibα chain at its carboxyl terminus.

11. The chimeric protein according to Claim 10, wherein the immunoglobulin molecule is derived from mouse.

12. A kit for measuring glycocalicin based on inhibition of a reaction of von Willebrand factor and glycoprotein Ib, comprising von Willebrand factor and a chimeric protein that consists of an Fc region of immunoglobulin molecule fused at its amino terminus to a partial protein comprising a von Willebrand factor binding site of glycoprotein Ibα chain at its carboxyl terminus.

[Detailed description of the invention]

[0001]

[Technical field]

The present invention relates to substance with antithrombotic activity and method for detecting glycocalicin. More precisely, it relates to a method for detecting or measuring a substance that inhibits binding of von Willebrand factor and glycoprotein Ib, and means directly used for carrying out the method.

[0002]

[Background art]

The global number of patients with thromboses such as myocardial infarction, cerebral infarction and peripheral artery occlusive disease is very large, and these diseases are very significant diseases to be diagnosed and treated. Platelets play a fundamental role for the onset of these thromboses. In general, if vascular endothelial cells in blood vessel cavities are impaired by arteriosclerotic lesion or the like, platelets will adhere to the impaired region to cause activation, and thus there are formed thrombocytic thrombi, which eventually develop into occlusive lesions.

[0003]

As one of the methods for detecting activation of platelets, there is a method of measuring glycocalicin concentration in plasma. Glycocalicin is a protein consisting of an enzymatically cleaved extracellular portion of a membrane glycoprotein present on surfaces of platelets, glycoprotein Ibα chain, and has a molecular weight of about 135 kDa. It is known that glycocalicin concentration in plasma is increased by impairment or activation of platelets, and it is currently used as a marker for detecting presence or absence of thrombotic diseases

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in clinical diagnosis. (J.H. Beer et al., *Blood*, *83*, 691-702, 1994; S. Kunishima et al., *Clin. Chem.*, *37*, 169-172, 1991).

[0004]

Many measurement methods of glycocalicin concentration have been reported, and any of these are based on ELISA (enzyme-linked immunosorbent assay) technique, wherein glycocalicin is detected by the sandwiching method utilizing two kinds of monoclonal antibodies directed to glycocalicin (J.H. Beer et al., supra; S. Kunishima et al., supra). Briefly, first monoclonal antibodies are immobilized on a 96-well plate or the like as a solid phase, blocked with a protein such as bovine serum albumin (BSA), and then added with patient's plasma (or serum) to be measured. Glycocalicin specifically binds to the monoclonal antibodies immobilized on the solid phase. The plate is washed, and added with second monoclonal antibodies labeled with an enzyme such as alkaline phosphatase and peroxidase or biotin so that the second antibodies should specifically bind to the glycocalicin bound to the first monoclonal antibodies. After washing, the plate is added with a substrate that can be converted into a substance exhibiting specific absorbance in a UV or visible region, fluorescence or luminescence with the enzyme used as the label of the second antibodies to perform an enzymatic reaction. Since the amount of glycocalicin in the patient's plasma and the binding amount of the second antibodies show positive correlation, the concentration of glycocalicin in the patient's plasma can be measured by quantifying the reaction product produced by the enzymatic reaction. A measurement method for glycocalicin by competitive ELISA utilizing one kind of anti-glycocalicin

antibodies has also been reported (H. Bessos *et al.*, *Thromb. Res.*, *59*, 497-507, 1990). However, the $IC_{50}$ value of the glycocalicin concentration showing competitive inhibition is about 4 μg/ml, and this makes the above measurement unusable for the measurement of the glycocalicin concentration in plasma (it is about 2 μg/ml in a healthy subject, J.H. Beer *et al.*, supra).

[0005]

The aforementioned glycocalicin quantification methods based on the sandwich technique are widely used at present. However, when a similar measurement system is desired to be newly prepared, it is necessary to obtain two kinds of anti-glycocalicin monoclonal antibodies having different recognition sites. Commercially available monoclonal antibodies are generally very expensive, and the preparation of monoclonal antibodies requires much labor such as acquisition of glycocalicin for immunization, acquisition of hybridoma from a spleen of immunized mouse and screening of a monoclonal antibody-producing cell. Further, it is impossible to measure an absolute value of glycocalicin concentration from the amount of the enzymatic reaction in the aforementioned sandwich ELISA method, and in many cases, it is necessary to measure glycocalicin of several kinds of known concentrations to obtain a calibration curve, and then it is necessary to calculate a concentration in a test sample to be measured based on comparison with the calibration curve. Therefore, it is important to establish a method capable of measuring an absolute concentration of glycocalicin in a simple manner without the complicated preparation of monoclonal antibodies, from a viewpoint of wide use in clinical diagnosis.

[0006]

Further, in an early stage of onset of thrombosis, von Willebrand factor in blood binds to subendothelial tissues (collagen etc.) exposed due to impairment of vascular endothelial cells, and the membrane glycoprotein, glycoprotein Ib, on platelets binds to the von Willebrand factor. Thus, the platelets adhere to blood vessel walls, and they are activated (J.P. Cean *et al.*, *J. Lab. Clin. Med.*, *87*, 586-596, 1976; K.J. Clemetson *et al.*, *Thromb. Haemost.*, *78*, 266-270, 1997). Therefore, it is an important target of antithrombotic drugs for treating or preventing thromboses to inhibit the binding of von Willebrand factor and glycoprotein Ib. However, there are few substances that have been proven to exhibit antithrombotic property by inhibiting the binding of the both proteins.

[0007]

It has been reported that a recombinant protein VCL that has a sequence of from the 504th to 728th amino acid residues of von Willebrand factor shows an antithrombotic action by inhibiting the binding of von Willebrand factor and glycoprotein Ib (K. Azzam *et al.*, *Thromb. Haemost.*, *73*, 318-323, 1995). Further, it has also been reported that a monoclonal antibody AJvW-2 directed to human von Willebrand factor exhibits an antithrombotic activity by specifically binding to von Willebrand factor without showing hemorrhagic tendency (S. Kageyama *et al.*, *Br. J. Pharmacol.*, *122*, 165-171, 1997; WO 96/17078). Furthermore, the protein AS1051 derived from snake venom specifically binds to the platelet glycoprotein Ib to similarly exhibit an antithrombotic property without showing hemorrhagic tendency (N. Fukuchi *et al.*, WO 95/08573).

Further, aurintricarboxylic acid, which is a

pigmental compound, has been reported to show an activity for inhibiting the binding of von Willebrand factor and glycoprotein Ib (M.D. Phiillips et al., *Blood*, 72, 1989-1903, 1988). However, it is known that its binding specificity is not high (K. Azzam et al., *Thromb. Haemost.*, *75*, 203-210, 1996; D. Mitra et al., *Immunology*, *87*, 581-585, 1996; R.M. Lozano et al., *Eur. J. Biochem.*, *248*, 30-36, 1997), and that the inhibition activity is exhibited by a polymerized macromolecule fraction (M. Weinstein et al., *Blood*, 78, 2291-2298, 1991; Z. Gua et al., *Thromb. Res.*, *71*, 77-88, 1993; H. Matsuno et al., *Circulation*, *96*, 1299-1304, 1997) etc.
[0008]

As described above, although it is an important target of antithrombotic drugs to inhibit the binding of von Willebrand factor and glycoprotein Ib, there is no low molecular weight compound that has reported to inhibit the binding of the both and have an antithrombotic activity, and therefore it is important to find out such a substance for attempting treatment and prevention of thromboses.
[0009]

The binding of von Willebrand factor and glycoprotein Ib is not observed under a usual condition, and it is considered that it occurs only under a condition where shear stress is induced in a blood flow (T.T. Vincent et al., *Blood*, 65, 823-831, 1985). However, as a method for artificially making it possible to observe the binding of the both proteins, there are known addition of an antibiotic, ristocetin (M.A. Howard and B.G. Firkin, *Thromb. Haemost.*, *26*, 362-369, 1971), and addition of a protein derived from snake venom, botrocetin (M.S. Read et al., *Proc. Natl. Acad. Sci. USA.*, 75, 45.14-4518, 1978). That is, the both substances are known

as a substance that binds to a specific site of von
Willebrand factor to cause a structural change of
the von Willebrand factor, thereby causing the
binding of the von Willebrand factor and
glycoprotein Ib, which does not occur under a usual
condition. As a method for observing the binding of
the both proteins, there is the following method
reported by Fujimura et al. (Y. Fujimura *et al.*,
*Blood*, 77, 113-120, 1991).
[0010]

That is, human von Willebrand factor is labeled
with ^{125}I in a conventional manner, and allowed to
bind to formalin-fixed platelets in the presence of
a certain amount of ristocetin or botrocetin. This
binding occurs due to the specific binding of the
von Willebrand factor to glycoprotein Ib on the
surfaces of the immobilized platelets, and after
unbound von Willebrand factor are removed by washing,
the amount of the both proteins bound to each other
can be measured by measuring the amount of [125]I.
Miura et al. detected the binding of the both
proteins by a similar method, wherein platelets were
immobilized on a 96-well plate via immobilized anti-
platelet membrane protein antibodies instead of the
use of formalin-fixed platelets (S. Miura *et al.*,
*Anal. Biochem.*, *236*, 215-220, 1996). Further,
Matsui et al. reported a method of binding
glycocalicin, which is a partial protein of the
extracellular portion of glycoprotein Ibα chain in
the presence of botrocetin, to von Willebrand factor
bound to collagen immobilized as a solid phase (T.
Matsui *et al.*, *J. Biochem.*, *121*, 376-381, 1997).
Furthermore, Moriki et al. produced a recombinant
protein expressing cell that expressed glycoprotein
Ib on the membrane, and reported that ^{125}I-labeled
von Willebrand factor bound to the glycoprotein Ib

on the membrane in the presence of botrocetin.
Moriki et al. further produced a cell expressing
glycoprotein Ib having a mutation in the amino acid
sequence, which bound to von Willebrand factor
without any inducing agent, and performed a binding
experiment. However, the binding amount was very
small compared with the binding amount in the
presence of botrocetin or ristocetin (T. Moriki et
al., *Blood*, *90*, 698-705, 1997).
[0011]

     As described above, all of the methods reported
so far for detecting the binding of von Willebrand
factor and glycoprotein Ib with high sensitivity
without shearing force are exclusively methods
utilizing addition of a binding inducing substance
such as botrocetin or ristocetin to a liquid phase.
However, the amount of botrocetin or ristocetin
changes the amount of the binding of von Willebrand
factor and glycoprotein Ib. Moreover, if a large
number of binding experiments are performed by using
a 96-well plate, for example, these methods
utilizing addition of the inducing substance to the
liquid phase are laborious. Furthermore, when the
aforementioned low molecular weight substance
inhibiting the binding of von Willebrand factor and
glycoprotein Ib is searched, an extremely large
number of binding experiments must be performed, and
therefore it is necessary from this viewpoint to
solve the aforementioned problem.
[0012]

[Problem to be solved by the invention]

     The problems of the aforementioned technical
background are summarized in the following three
points.
[0013]

     (1) Although methods for quantification of

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glycocalicin is important for diagnosis of
thromboses, conventional highly sensitive methods
are sandwich ELISA methods. Therefore, two kinds of
monoclonal antibodies having different recognition
sites are required, and a calibration curve prepared
with a standard substance is required for the
quantification.
[0014]

    (2) It is important to discover a low molecular
weigh inhibition substance for the binding of von
Willebrand factor and glycoprotein Ib, and use it as
a drug, in view of the treatment and prevention of
thromboses. However, no low molecular weight drug
has been known so far, which targets the inhibition
of the binding of von Willebrand factor and
glycoprotein Ib and is reported to have
antithrombotic activity.
[0015]

    (3) In order to find out such a drug as
mentioned in the above (2), large number of binding
inhibition experiments must be performed for von
Willebrand factor and glycoprotein Ib. However, the
methods comprising addition of a binding inducing
substance to a liquid phase are complicated, and
have problems concerning accuracy.
[0016]

    The present invention has been accomplished
from the aforementioned viewpoints, and an object of
the present invention is to provide a method for
detecting the binding of von Willebrand factor and
glycoprotein Ib in a simple manner, a simple method
for measurement of glycocalicin, and a simple method
for measurement of a substance that can be an
antithrombotic drug of which working point is the
inhibition of the binding of von Willebrand factor
and glycoprotein Ib, as well as means for use in

these measurement methods.

[0017]

[Means to solve the problem]

The inventors of the present invention assiduously studied in order to achieve the aforementioned object. That is, a protein expression system based on an animal cell was prepared first for obtaining a chimeric molecule consisting of a partial protein of glycoprotein Ibα chain bound to the Fc region of immunoglobulin molecule (hereinafter referred to as "chimeric protein"). Further, they found that, if von Willebrand factor was immobilized in the presence of botrocetin, the aforementioned chimeric protein, i.e., glycoprotein Ib molecule, specifically bound to the immobilized von Willebrand factor without a binding inducing substance in a liquid phase, and that a binding test can be performed in a simple manner to measure the binding amount by labeling commercially available inexpensive anti-immunoglobulin Fc antibodies or directly labeling the chimeric protein, and thus accomplished the present invention.

[0018]

That is, the first method according to the present invention is a method for detecting binding of von Willebrand factor and glycoprotein Ib or inhibition of the binding, comprising the steps of immobilizing von Willebrand factor in a reaction vessel in the presence of a substance inducing the binding of von Willebrand factor and glycoprotein Ib, and, reacting the immobilized von Willebrand factor with glycoprotein Ib.

[0019]

The second method according to the present invention is a method for detecting binding of von

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Willebrand factor and glycoprotein Ib or inhibition of the binding, comprising the steps of binding a chimeric protein that consists of an Fc region of immunoglobulin molecule fused at its amino terminus to a partial protein comprising a von Willebrand factor binding site of glycoprotein Ibα chain at its carboxyl terminus or the chimeric protein labeled with a labeling substance to von Willebrand factor immobilized in a reaction vessel, and detecting the Fc region of the immunoglobulin molecule or the labeling substance.

[0020]

As the substance that induces the binding of von Willebrand factor and glycoprotein Ib, botrocetin can be mentioned.

Further, in another embodiment of the second method, von Willebrand factor is immobilized in the reaction vessel in the presence of a substance that induces the binding of von Willebrand factor and glycoprotein Ib.

[0021]

In the first and second methods, glycocalicin contained in a sample can be measured by adding the sample to the reaction vessel during the reaction of von Willebrand factor and glycoprotein Ib, or prior to the reaction, and detecting inhibition of the binding of von Willebrand factor and glycoprotein Ib.

[0022]

Further, in the first and second methods, a substance that inhibits the binding of von Willebrand factor and glycoprotein Ib can be detected by adding a sample containing a substance to be detected to the reaction vessel during the reaction of von Willebrand factor and glycoprotein Ib, or prior to the reaction, and detecting inhibition of the binding of von Willebrand factor

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and glycoprotein Ib.
[0023]

The present invention further provides a chimeric protein, which consists of an Fc region of immunoglobulin molecule fused at its amino terminus to a partial protein comprising a von Willebrand factor binding site of glycoprotein Ibα chain at its carboxyl terminus.
[0024]

The present invention also provides a kit for measuring glycocalicin based on inhibition of a reaction of von Willebrand factor and glycoprotein Ib, which comprises von Willebrand factor and a chimeric protein that consists of an Fc region of immunoglobulin molecule fused at its amino terminus to a partial protein comprising a von Willebrand factor binding site of glycoprotein Ibα chain at its carboxyl terminus.
[0025]

The term "chimeric protein" used in the present specification means a chimeric protein that consists of an Fc region of immunoglobulin molecule fused at its amino terminus to a partial protein comprising a von Willebrand factor binding site of glycoprotein Ib at its carboxyl terminus. Further, the term "glycoprotein Ib" used for the methods of the present invention may refer to glycoprotein Ib itself or the chimeric protein, or the both of them.

The term "detection" used in the present specification mainly means finding out a substance or a phenomenon, but it may also mean measurement of amount of the substance or degree of the phenomenon as a result of the finding of the substance or the phenomenon. Further, the term "measurement" mainly means measurement of an amount of substance or a degree of phenomenon, but it may also mean finding

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out the substance or the phenomenon.
[0026]
[Embodiments of the invention]

The present invention will be explained in detail hereinafter.
[0027]
<1> Chimeric protein

The chimeric protein of the present invention is a protein consisting of a partial protein comprising a von Willebrand factor binding site of glycoprotein Ibα chain, which is one of platelet membrane proteins of human or other mammals, bound to an Fc region of a heavy chain (H chain) of immunoglobulin molecule of mouse, human or other mammals by means of a genetic engineering technique. This chimeric protein can be produced by using cultured cells. In the chimeric protein, the partial protein comprising von Willebrand factor binding site of glycoprotein Ibα chain and the Fc region of the immunoglobulin molecule are bound at the carboxyl terminus of the partial protein and the amino terminus of the Fc region.
[0028]

As an example of the partial protein of glycoprotein Ibα chain, there can be mentioned a partial protein having a sequence comprising amino acid residues of from the amino terminus to the aspartic acid residue at the 319th position (amino acid numbers 1-319) of glycoprotein Ibα chain molecule. However, since it is considered that von Willebrand factor binding site is a region contained in the amino acid sequences of the amino acid numbers 1-293 (V. Vincente *et al.*, *J. Biol. Chem.*, *263*, 18473-18479, 1988), and in the sequence of the amino acid numbers 251-285 (V. Vicente *et al.*, *J. Biol. Chem.*, *265*, 274-280, 1990), a partial protein

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containing at least these regions may be sufficient.
[0029]
　　　Further, the Fc region of immunoglobulin
molecule may be derived from any animals, and may be
of any subtype, and those that can be purified
and/or detected with commercially available
polyclonal antibodies and/or monoclonal antibodies,
protein A, protein G or the like may be used. The
immunoglobulin heavy chain comprises regions called
VH domain, CH1 domain, hinge domain, CH2 domain and
CH3 domain (and further CH4 domain in IgE) connected
in this order from the amino terminus.
　　　For example, the Fc region used for the
chimeric protein may be a continuous sequence from
the hinge domain to the CH3 domain of the above
sequence. However, from the viewpoint that it
should be able to be purified and/or detected with
commercially available polyclonal antibodies and/or
monoclonal antibodies, protein A, protein G or the
like, the hinge domain is not essential, and it may
partially contain a mutation such as deletion and
insertion of one or more amino acid residues.
Further, while the immunoglobulin may be derived
from any animals including human and mouse, one
derived from mouse can be used, for example.
Although the subtype of the immunoglobulin may be
any subtype, IgG can be used, for example. The
subclass may also be any subclass, and IgG1, IgG2a
and so forth can be mentioned, for example.
Exemplary amino acid sequences of the chimeric
protein of the present invention are shown in SEQ ID
NOS: 7 and 14. In SEQ ID NOS: 7 and 14, it is
presumed that 16 amino acid residues at the N-
terminus constitute a signal peptide.
[0030]
　　　The chimeric protein of the present invention

can be produced by allowing expression of a chimeric
gene coding for it (chimeric protein gene) in a
suitable cell. A chimeric protein gene can be
prepared by obtaining a glycoprotein Ibα chain gene
and an immunoglobulin heavy chain gene respectively
from a cDNA library, genomic library, DNA fragment
or the like using genetic engineering techniques or
chemically preparing them, and ligating them.
[0031]

A glycoprotein Ibα chain gene can be obtained
from, for example, a cDNA library produced by using
a phage vector or the like from mRNA of HEL cell,
which is a human megakaryocyte cell strain, through
reverse transcription PCR using suitable primer DNA
designed based on a known DNA sequence of
glycoprotein Ibα chain gene. Further, a clone
containing a glycoprotein Ibα chain gene can be
obtained from such a cDNA library by performing
hybridization using a probe DNA designed based on
the known DNA sequence. Alternatively, it can be
obtained by excising it from plasmid containing a
glycoprotein Ibα chain gene registered at ATCC
(American Type Culture Collection, pGPIb2.4,
deposition number: ATCC65755) with a suitable
restriction enzyme.
[0032]

A gene of immunoglobulin heavy chain can be
obtained from, for example, cDNA library produced
from mRNA of mouse immunoglobulin producing
hybridoma by using a phage vector or the like
through reverse transcription PCR using suitable
primer DNA designed based on a known DNA sequence of
the immunoglobulin heavy chain gene. Further, a
clone containing a mouse immunoglobulin gene can be
obtained from such a cDNA library by performing
hybridization using a probe DNA designed based on

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the known DNA sequence.
[0033]

A chimeric protein gene can be obtained by digesting DNA strands of a full length glycoprotein Ibα chain gene or a partial sequence thereof and a full length mouse immunoglobulin heavy chain γ1 gene or γ2a gene or a partial sequence thereof with a suitable restriction enzyme and then ligating them. The digestion and the ligation of the both genes may be performed so that the ligation product should code for a chimeric protein consisting of an Fc region of immunoglobulin molecule fused at its amino terminus to a partial protein comprising a von Willebrand factor binding site of glycoprotein Ibα chain at its carboxyl terminus. Further, extracellular secretion of the chimeric protein is desired, the segment for glycoprotein Ibα chain may contain a signal peptide.
[0034]

A chimeric protein gene produced as described above is expressed by using a suitable host-vector system. As the host, animal cells, insect cells and so forth can be mentioned. The vector is not particularly limited so long as it can function as a vector in the host cell, and it is preferable to use an expression vector having a promoter suitable for the host cell. A chimeric protein can be produced by transforming the host cell with a recombinant vector obtained by inserting a chimeric protein gene into an expression vector, and culturing the transformed cell.
[0035]

While a chimeric protein produced as described above may be used as it is, it can be readily purified by utilizing the Fc region of immunoglobulin molecule through affinity

chromatography using immobilized protein A, protein G, anti-immunoglobulin antibodies and so forth.
[0036]
<2> Method for detecting binding of von Willebrand factor and glycoprotein Ib or inhibition of this binding

The first method for detecting the binding of von Willebrand factor and glycoprotein Ib or inhibition of the binding according to the present invention is characterized in that von Willebrand factor is immobilized in a reaction vessel in the presence of a substance inducing the binding of von Willebrand factor and glycoprotein Ib (henceforth also referred to as "binding inducing substance"), and the immobilized von Willebrand factor is allowed to react with the glycoprotein Ib.
[0037]

By immobilizing von Willebrand factor in a reaction vessel in the presence of a binding inducing substance, a step of adding a binding inducing substance during the reaction of von Willebrand factor and glycoprotein Ib in a liquid phase can be omitted.
[0038]

von Willebrand factor can be prepared from human blood according to the method described in H.R. Gralnick et al., J. Clin. Invest., 62, 496 (1978) or the like.

As the binding inducing substance, there can be mentioned botrocetin, ristocetin and so forth, and botrocetin is preferred.
[0039]

As the reaction vessel in which von Willebrand factor is immobilized, a vessel made of synthetic resin such as polystyrene and polycarbonate or glass may be used. More specifically, a 96-well multi-

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well plate made of polystyrene and so forth can be mentioned. By injecting a solution containing von Willebrand factor into the aforementioned reaction vessel, von Willebrand factor can be immobilized on a wall surface of the vessel. It is also possible to immobilize collagen on a wall surface of the reaction vessel, and allow von Willebrand factor to bind to the collagen. The conditions for immobilizing von Willebrand factor or collagen to a reaction vessel are not particularly limited so long as they can be immobilized. However, when a vessel made of polystyrene is used, for example, it is preferable to use a neutral solution, preferably at pH 6.8-7.8, more preferably at about pH 7.4.
[0040]

For the immobilization of von Willebrand factor, while a solution containing von Willebrand factor and a solution containing a binding inducing substance may be separately added to a reaction vessel, it is preferable to prepare a solution containing both of von Willebrand factor and a binding inducing substance, and add it into the reaction vessel, from the viewpoint of operation efficiency. Further, a reaction vessel in which von Willebrand factor is immobilized is preferably added with a bovine serum albumin solution or the like to block unbound areas on the wall surface.
[0041]

After von Willebrand factor is immobilized in a reaction vessel, the reaction vessel is washed and then glycoprotein Ib is added. Upon addition of glycoprotein Ib, the biding reaction of von Willebrand factor and glycoprotein Ib is caused. This reaction is attained in a liquid phase. Subsequently, the binding of von Willebrand factor and glycoprotein Ib is detected. This detection can

be performed by the method usually used for detection of the binding of von Willebrand factor and glycoprotein Ib.

[0042]

The second method according to the present invention is a method wherein the binding of von Willebrand factor and glycoprotein Ib or inhibition of the binding is detected by allowing the aforementioned chimeric protein or the chimeric protein labeled with a labeling substance to bind to von Willebrand factor immobilized in a reaction vessel, and detecting the Fc region of the immunoglobulin molecule or the labeling substance. More specifically, a solution containing von Willebrand factor is added to the reaction vessel to immobilize the von Willebrand factor on a wall surface of the reaction vessel. Then, a solution containing a chimeric protein is added to the reaction vessel to allow the chimeric protein to bind to the immobilized von Willebrand factor. This binding can be induced by the presence of a binding inducing substance in the reaction system of the von Willebrand factor and the chimeric protein. Specifically, von Willebrand factor is immobilized in the reaction vessel in the presence of a binding inducing substance in a manner similar to that of the aforementioned first method, or the binding inducing substance is added at the same time as, or at a time point around the addition of the solution containing the chimeric protein to the reaction vessel.

[0043]

The chimeric protein binds to the immobilized von Willebrand factor at the von Willebrand factor binding site of glycoprotein Ib contained in the molecule. The detection of the chimeric protein

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bound to von Willebrand factor as described above
can be performed by, for example, detecting the Fc
region of the immunoglobulin molecule contained in
the molecule. For the detection of the Fc region, a
method usually used for immunoassay can be used.

Specifically, for example, a labeled substance
that specifically binds to the Fc region such as
protein A, protein G, and anti-immunoglobulin
antibodies is added to the reaction vessel, and the
label is detected. As the labeling substance, there
can be mentioned enzymes such as alkaline
phosphatase and peroxidase, biotin, avidin,
fluorescent substances such as fluorescein,
compounds containing a fluorescent rare earth
element such as europium and lanthanoids and so
forth. Biotin or avidin is detected by further
binding to them another labeling substance bound to
avidin or biotin. Enzymes can be detected by adding
a suitable substrate to cause an enzymatic reaction
and observing visible absorbance, UV absorbance,
fluorescence, luminescence etc. Furthermore,
fluorescent substances and compounds having a
property of emitting fluorescence can be detected
based on fluorescence emitted upon irradiation with
excitation light.
[0044]

The chimeric protein bound to the immobilized
von Willebrand factor can also be detected by using
a chimeric protein labeled with a labeling substance
beforehand and detecting this labeling substance.
The labeling substance and the detecting method
therefor may be similar to those mentioned above for
use in the detection of the Fc region. When a
chimeric protein labeled with a labeling substance
is used, a purified chimeric protein is preferably
used.

The purification of the chimeric protein can be attained by using the Fc region of the immunoglobulin molecule through affinity chromatography and so forth as described above.
[0045]

In the aforementioned first and second methods, inhibition of the binding of von Willebrand factor and glycoprotein Ib can be detected by comparing a case where a substance inhibiting the binding of von Willebrand factor and glycoprotein Ib (henceforth also referred to as "binding inhibition substance") is added to a reaction vessel at substantially the same time as the addition of glycoprotein Ib (or a chimeric protein) to the reaction vessel or prior to the addition of glycoprotein Ib and a case where the inhibition substance is not added for the binding of von Willebrand factor and glycoprotein Ib.
[0046]

Further, in the aforementioned methods, a substance inhibiting the binding of von Willebrand factor and glycoprotein Ib can be detected by adding a sample containing a substance to be detected to the reaction vessel during the reaction of von Willebrand factor and glycoprotein Ib or prior to the reaction and detecting inhibition of the binding of von Willebrand factor and glycoprotein Ib. If a standard curve that represents the relation between amount of an inhibition substance and binding of von Willebrand factor and glycoprotein Ib is prepared, the inhibition substance of an unknown amount can be quantified.
[0047]

No low molecular weight compound has been reported so far, which inhibits the binding of von Willebrand factor and glycoprotein Ib and has antithrombotic activity. The methods of the present

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invention are extremely simpler compared with the
conventional methods, and are also useful for search
of such a low molecular compound as mentioned above.
[0048]
<3> Method and kit for measurement of glycocalicin
     In the aforementioned first and second methods,
glycocalicin can be measured by adding a sample
containing glycocalicin to the reaction vessel
during the reaction of von Willebrand factor and
glycoprotein Ib or prior to the reaction and
detecting inhibition of the binding of von
Willebrand factor and glycoprotein Ib.  If a
standard curve that represents the relation between
glycocalicin concentration and the binding of von
Willebrand factor and glycoprotein Ib is prepared,
concentration of glycocalicin in an unknown amount
can be measured.
[0049]
     If von Willebrand factor and the chimeric
protein are prepared as a kit, the measurement of
glycocalicin according to the present invention can
conveniently be performed.  As such a kit, there can
be specifically exemplified a kit comprising von
Willebrand factor, a chimeric protein, a binding
inducing substance, glycocalicin of a known amount,
anti-immunoglobulin antibodies labeled with alkaline
phosphatase or the like, a reagent for detecting the
label and a washing buffer.  As another embodiment,
there can be exemplified a kit comprising von
Willebrand factor, a chimeric protein labeled with a
labeling substance, a binding inducing substance,
glycocalicin of a known amount, a reagent for
detecting the label and a washing buffer.
[0050]
[Examples]
     The present invention will be explained more

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specifically in to the following examples.
[0051]
[Example 1] Preparation of chimeric protein gene
<1> Cloning of glycoprotein Ibα chain gene

Cloning of human glycoprotein Ibα chain gene
was attained by constructing a cDNA library from
human erythroleukemia cells (HEL) according to the
method described in Molecular Cloning (Sambrook, J.,
Fritsch, E.F., Maniatis, T., Molecular Cloning, Cold
Spring Harbor Laboratory Press (1989)). That is,
human erythroleukemia cells were stimulated by
culturing them in a medium containing 160 nM of a
phorbol ester (phorbol-12-myristate-13-acetate: PMA)
for 48 hours, and then the medium was removed. A
guanidinium thiocyanate buffer (4.0 M guanidinium
thiocyanate, 0.1 M Tris-HCl (pH 7.5), 1% 2-
mercaptoethanol) was added to the cells to suspend
the cells in the buffer. The cell suspension was
subjected to disruption treatment by using a
Polytron homogenizer (produced by Brinkmann).
[0052]

Laurylsarcosinate (sodium laurylsarcosinate)
was added to the disrupted cell suspension at a
final concentration of 0.5%. This solution was
centrifuged at 5000 x g for 10 minutes to remove the
precipitates. The centrifugation supernatant was
overlaid on cesium chloride/EDTA solution (5.7 M
CsCl, 0.01 M EDTA, pH 7.5) contained in an
ultracentrifugation tube and subjected to
ultracentrifugation at 100000 x g for 20 hours. The
precipitated RNA was collected and purified by
ethanol precipitation to obtain total RNA.
[0053]

The obtained total RNA was loaded on an oligo-
dT cellulose column to obtain mRNA. From 10 μg of
this mRNA, single-stranded DNA was prepared by using

random hexamer oligo DNA as a primer and a reverse transcriptase, and then double-stranded cDNA was prepared by using a DNA polymerase. An EcoRI adapter was ligated to this cDNA by using T4 DNA ligase. The cDNA to which the adapter was ligated was subjected to a phosphorylation treatment using T4 polynucleotide kinase, and purified by using a gel filtration column. A λgt10 arm prepared so that it could be inserted into an EcoRI restriction site (produced by Stratagene) was ligated to this DNA using T4 DNA ligase. This recombinant DNA was packaged in phage to obtain a cDNA library.
[0054]

Escherichia coli NM514 was infected with this phase. Plaque hybridization was performed for the produced phage plaques by using oligo DNA (SEQ ID NO: 1) end-labeled with a radioisotope ($^{32}$P) as a probe. That is, the produced phage plaques were transferred to a nitrocellulose filter, and DNA was denatured with an alkaline denaturation solution (0.5 M sodium hydroxide, 1.5 M sodium chloride). The filter was neutralized with a neutralization solution (0.5 M Tris-HCl, pH 7.0, 1.5 M sodium chloride), and heated at 80°C for 2 hours to immobilize the DNA on the filter. Synthesized DNA (chemically synthesized by using a DNA synthesizer Model 380A produced by Perkin-Elmer Applied Biosystems) was labeled at the 5' end of the DNA with γ-$^{32}$P-ATP with the aid of T4 DNA kinase (produced by Takara Shuzo), and used as probe DNA. The nucleotide sequence of the aforementioned oligo DNA was designed based on the nucleotide sequence of a known human glycoprotein Ibα chain gene (J.A. Lopez et al., Proc. Natl. Acad. Sci. USA, 84, 5615-5619 (1987)).
[0055]

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The nitrocellulose filter (diameter: 132 mm) on which phage plaque DNA was transferred was immersed in 4 ml of a hybridization buffer (0.9 M sodium chloride, 0.09 M sodium citrate (pH 7.0), 0.5% sodium laurylsulfate, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 100 µg/ml heat-denatured salmon sperm DNA) containing the probe corresponding to 1 x $10^6$ cpm (count per minute) per one filter, and allowed to hybridize at 42°C for 16 hours.  The filter was washed three times with 1 x SSC (0.875% sodium chloride, 0.441% sodium citrate, pH 7.0) and 0.1% sodium laurylsulfate solution at 37°C for 30 minutes to remove the probe non-specifically adsorbed on the filter.  After the filter was dried, radioautography was performed by using an X-ray film.  As a result, four strains of positive clones were obtained.
[0056]

The phage was isolated form each positive clone, and *Escherichia coli* NM514 was infected with the isolated phage and proliferated.  Then, phage DNA from each clone was purified by cesium chloride density gradient ultracentrifugation.  This phage DNA was digested with a restriction enzyme *Eco*RI, and DNA was purified by agarose electrophoresis. This purified DNA was inserted into the *Eco*RI site of pBluescriptSK- (produced by Stratagene) and used for transformation of the *Escherichia coli* XLIIblue (produced by Stratagene) to obtain a transformant. Plasmid was prepared from the transformant by the alkali SDS method, and the nucleotide sequence of the plasmid DNA was determined by the dideoxy method using a DNA sequencer Model 377 produced by Perkin-Elmer Applied Biosystems according to the protocol attached to the instrument.  It was confirmed that one strain among the obtained positive clones

contained cDNA of 2.4 kb, and it was the clone
having the full length of human glycoprotein Ibα
gene reported by J.A. Lopez *et al.* (*Proc. Natl. Acad.
Sci. USA*, Vol. *84*, pp.5615-5619 (1987)). This
plasmid was designated as pBluescriptGPIbAlpha.
[0057]
<2> Cloning of gene coding for Fc region of
immunoglobulin (γ1 origin)

     The gene for the Fc region of mouse
immunoglobulin γ1 was obtained by extracting total
RNA from a mouse hybridoma cell strain MB40.3 and
performing reverse transcription PCR. That is, from
10 ml of culture broth of MB40.3 cells, the cells
were collected by centrifugation, and the cells were
lysed with ISOGEN (1 ml, produced by Nippon Gene).
The lysate was subjected to syringing using an
injection needle of 18G. The lysate was left for 5
minutes, then added with 200 μl of chloroform and
mixed. The mixture was left stand for 2 minutes and
then centrifuged (15000 rpm, 15 minutes) to recover
an aqueous phase. The aqueous phase was added with
500 μl of 2-propanol, mixed, left stand for 5
minutes and centrifuged (15000 rpm, 15 minutes) to
precipitate the total RNA. The total RNA was washed
with 75% ethanol and dissolved in 100 μl of
sterilized water.
[0058]

     cDNA was prepared by using 3 μg (20 μl) of
MB40.3 cell total RNA prepared as described above as
a template and using random primers and reverse
transcriptase (Superscript II produced by GIBCO).
The cDNA was amplified by PCR using the primers of
SEQ ID NOS: 2 and 3, digested with HindIII and BamHI,
purified by agarose gel electrophoresis, and ligated
to pGEM-3Zf (produced by Promega) digested with
*Hind*III and *Bam*HI. *Escherichia coli* XLIIblue

(produced by Stratagene) was transformed with the obtained recombinant DNA. One of the obtained transformants was cultured. Plasmid was prepared by the alkali SDS method, and the nucleotide sequence thereof was determined by the dideoxy method using a DNA sequencer Model 377 produced by Perkin-Elmer Applied Biosystems according to the protocol attached to the instrument. The obtained nucleotide sequence of the gene fragment for the Fc region of mouse immunoglobulin γ1 is shown in SEQ ID NO: 4. This plasmid was designated as pGEMmIgG1Fc.
[0059]
<3> Preparation of plasmid expressing chimeric protein (GPIb-mIgG1Fc)

A chimeric protein comprising the human glycoprotein Ib gene and the Fc region of mouse immunoglobulin γ1 obtained as described above, which were fused together, was prepared as follows.

First, the plasmid pBluescriptGPIAlpha containing the glycoprotein Ibα chain gene was digested with restriction enzymes EcoRI and XbaI, and separated by agarose gel electrophoresis to recover DNA of about 1000 bp, which corresponded to the N-terminus region of glycoprotein Ibα chain gene. This was inserted into the EcoRI-XbaI site of pBluescriptSK- (produced by Stratagene) to prepare plasmid pBluescriptGPIbEX.
[0060]

Separately, the plasmid pGEMmIgG1Fc containing the partial gene of mouse immunoglobulin γ1 obtained as described above was digested with a restriction enzyme XbaI and separated by agarose gel electrophoresis to recover the IgG1Fc gene of 700 bp. This DNA was ligated to pBluescriptGPIbEX digested with a restriction enzyme XbaI and subjected to CIAP treatment to obtain plasmid pBluescriptGPIbIgG1FcFH.

The protein encoded by this gene was designated as GPIb-mIgG1Fc, of which gene sequence and amino acid sequence are shown in SEQ ID NOS: 6 and 7, respectively.  In SEQ ID NO: 6, it is presumed that the 16 amino acid residues of the N-terminus constitute a signal peptide.
[0061]

Further, pBluescriptGPIbIgG1FcFH was digested with a restriction enzyme XhoI, and DNA coding for GPIbFcFH was separated by agarose gel electrophoresis.  This DNA was inserted into the XhoI site of an expression vector for animal cells pSD(X) to obtain an expression vector pSDGPIbIgG1FcFH, in which the GPIb gene was inserted downstream from a promoter.  The outline of the aforementioned procedure is shown in Fig. 1. Escherichia coli XLIIblue (Escherichia coli AJ13434) harboring the plasmid pSDGPIbIgG1FcFH was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry on April 2, 1998, and given an accession number of FERM P-16749.
[0062]
<4> Cloning of gene coding for Fc region of immunoglobulin (γ2a origin)

The gene of the Fc region of mouse immunoglobulin γ2a was obtained by extracting total RNA from a mouse hybridoma cell strain W6/32 and performing reverse transcription PCR.  That is, from 10 ml of culture broth of W6/32 cells, the cells were collected by centrifugation, and the cells were lysed with ISOGEN (1 ml, produced by Nippon Gene).  The lysate was subjected to syringing using an injection needle of 18G.  The lysate was left for 5 minutes, then added with 200 μl of chloroform and

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mixed. The mixture was left stand for 2 minutes and then centrifuged (15000 rpm, 15 minutes) to recover an aqueous phase. The aqueous phase was added with 500 µl of 2-propanol, mixed, left stand for 5 minutes and then centrifuged (15000 rpm, 15 minutes) to precipitate the total RNA. The total RNA was washed with 75% ethanol and dissolved in 100 µl of sterilized water.

[0063]

cDNA was prepared by using 3 µg (20 µl) of the W6/32 cell total RNA prepared as described above as a template and using random primers and reverse transcriptase (Superscript II produced by GIBCO). The cDNA was amplified by PCR using primers having nucleotide sequences of SEQ ID NOS: 8 and 9, digested with *Hind*III and *Bam*HI, purified by agarose gel electrophoresis, and ligated to pGEM-3Zf (produced by Promega) digested with *Hind*III and BamHI. *Escherichia coli* XLIIblue (produced by Stratagene) was transformed with the obtained recombinant DNA. One of the obtained transformants was cultured. Plasmid was prepared by the alkali SDS method and the nucleotide sequence thereof was determined by the dideoxy method using a DNA sequencer Model 377 produced by Perkin-Elmer Applied Biosystems according to the protocol attached to the instrument. The obtained nucleotide sequence of the gene fragment for the Fc region of mouse immunoglobulin γ2a is shown in SEQ ID NO: 10. This plasmid was designated as pGEMmIgG2aFc.

[0064]

<5> Preparation of plasmid expressing chimeric protein (GPIb-mIgG2aFc)

A chimeric protein gene comprising the human glycoprotein Ib gene and the Fc region of mouse immunoglobulin γ1 obtained as described above and

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fused together was prepared as follows.
[0065]

First, the plasmid pBluescriptGPIAlpha containing the glycoprotein Ibα chain gene was digested with restriction enzymes *Eco*RI and *Xba*I, and separated by agarose gel electrophoresis to obtain a *Kpn*I-*Xba*I DNA fragment containing the sequence of glycoprotein Ib gene for the sequence of from the N-terminus to the 319th aspartic acid.
[0066]

Further a gene fragment of the Fc region of mouse immunoglobulin γ2a having an *Xho*I site at the 5' end side and an *Xba*I site at the 3' end side was produced by PCR (annealing temperature: 55°C, 30 cycles) using the plasmid pGEMmIgG2aFc containing the partial gene of mouse immunoglobulin γ2a obtained as described above, two kinds of synthetic primers having the nucleotide sequences shown in SEQ ID NOS: 9 and 12 and PFU (produced by Stratagene). This gene fragment was digested with XbaI and XhoI, then purified by agarose gel electrophoresis and ligated to pBluescriptSK- digested with *Xba*I and *Xho*I. *Escherichia coli* XLIIblue (produced by Stratagene) was transformed with the obtained recombinant plasmid. Plasmid was prepared from the obtained transformant by the alkali SDS method, and the nucleotide sequence thereof was determined by the dideoxy method using a DNA sequencer Model 377 produced by Perkin-Elmer Applied Biosystems according to the protocol attached to the instrument. As a result, it was confirmed to have a nucleotide sequence corresponding to the nucleotide sequence shown in SEQ ID NO: 10 of which 6 nucleotides at the 5' end was replaced with TCTAGAC and 6 nucleotides at the 3' end was eliminated. This plasmid was designated as pBluescriptmIgG2a. This plasmid was

digested with *Xba*I and *Xho*I, and purified by agarose
gel electrophoresis to obtain a *Xba*I-*Xho*I fragment
of the Fc region gene of mouse immunoglobulin γ2a.
[0067]

The *Kpn*I-*Xba*I fragment of human glycoprotein Ib
gene and the *Xba*I-*Xho*I fragment of Fc region gene of
mouse immunoglobulin γ2a obtained as described above
were ligated to pBluescriptSK- digested with *Kpn*I
and *Xho*I, and *Escherichia coli* XLIIblue (produced by
Stratagene) was transformed with the obtained
recombinant plasmid.  One of the obtained
transformants was cultured, and plasmid was prepared
by the alkali SDS method to obtain plasmid
containing a gene coding for a protein (chimeric
protein) comprising the N-terminus side region of
glycoprotein Ib (amino acid numbers 1-319, including
a signal peptide) and the Fc region of mouse
immunoglobulin γ2a bound together (SEQ ID NO: 13).
This plasmid was designated as pBluescriptGPIbFc2a,
and the encoded chimeric protein corresponding to
the gene was especially designated as GPIb-mIgG2aFc,
of which amino acid sequence was shown in SEQ ID NO:
14.  In SEQ ID NO: 14, it is presumed that 16 amino
acid residues of the N-terminus constitute a signal
peptide.  *Escherichia coli* XLIIblue (*Escherichia
coli* AJ13432) harboring the plasmid
pBluescriptGPIbFc2a was deposited at the National
Institute of Bioscience and Human-Technology, Agency
of Industrial Science and Technology, Ministry of
International Trade and Industry on March 19, 1998,
and given an accession number of FERM P-16719.
[0068]

pBluescriptGPIbFc2a was digested with *Xho*I, and
purified by agarose electrophoresis.  This *Xho*I
fragment containing a gene of the chimeric protein
was ligated to the XhoI site of the same animal cell

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expression vector pSD(x) as the above <3> to obtain
plasmid pSDGPIbFc2a.  Further, pGPIbFcbluescript was
digested with *Eco*RI and *Xho*I, and the *Eco*RI-*Xho*I
fragment containing the chimeric protein gene was
inserted into the EcoRI-XhoI site of expression
vector pMikNeo(+) for animal cells (kindly provided
by Dr. K. Maruyama, the Institute of Medical Science,
the University of Tokyo) having SRα promoter (K.
Maruyama and Y. Takebe *et al.*, *Medical Immunology*,
*20*, 27-32, 1990) to obtain plasmid pMikGPIbFc.  The
outline of the procedure used for obtaining
pMikGPIbFc is shown in Fig. 2.
[0069]
[Example 2] Production of chimeric protein (GPIb-
mIgG1Fc) using animal cells

Cells producing the chimeric protein were
produced as follows.  CHOdhfr⁻ cells were cultured by
using D-MEM medium (10 ml, produced by GIBCO)
containing 10% fetal bovine serum at 37°C under 5%
$CO_2$ at a density of $5 \times 10^5$ cells per 10-cm dish.
The cells were transfected with pSDGPIbIgG1Fc
prepared as described in Example 1 <3>.  The
transfection was performed by using calcium
phosphate as described below.  That is, about 10 µg
per 10-cm dish of pSDGPIbIgG1Fc was added to 0.5 ml
of BES buffer (pH 6.96) containing 0.125 M calcium
chloride, uniformly added dropwise to a dish, and
incubated overnight at 35°C under 3% $CO_2$.  Then, the
dish was washed twice with PBS, and further
incubated in α-MEM medium not containing nucleic
acid at 37°C for about 24 hours under 5% $CO_2$.  The
cells transfected as described above were further
cultured in α-MEM medium not containing nucleic
acids, but containing 0.05 µM methotrexate (MTX) and
10% fetal bovine serum to obtain chimeric protein
producing cells.

[0070]

The chimeric protein producing cells obtained as described above were cultured in an F175 cell culture flask containing α-MEM medium not containing nucleic acid, but containing 0.05 μM methotrexate (MTX) and 10% fetal bovine serum, until about 60% confluent. Then, the medium was exchanged with a serum-free medium, ASF104 medium (produced by Ajinomoto), containing 0.05 μM methotrexate (MTX), and the culture supernatant was collected four days later.

[0071]

[Example 3] Production of chimeric protein (GPIb-mIgG2aFc) using animal cells

Cells producing the chimeric protein were produced as follows. CHOK1 cells were cultured by using D-MEM medium (10 ml, produced by GIBCO) containing 10% fetal bovine serum at 37°C under 5% $CO_2$ at a density of $5 \times 10^5$ cells per 10-cm dish. The cells were transfected with pMikGPIbFc prepared in Example 1. The transfection was performed by the calcium phosphate method as described below. That is, about 10 μg per 10-cm dish of pMikGPIbFc was added to 0.5 ml of BES buffer (pH 6.96) containing 0.125 M calcium chloride, uniformly added dropwise to a dish, and incubated overnight at 35°C under 3% $CO_2$. Then, the dish was washed twice with PBS, and further incubated in D-MEM medium at 37°C for about 24 hours under 5% $CO_2$. The cells transfected as described above were further cultured in D-MEM medium containing G418 (850 μg/ml) and 10% fetal bovine serum to obtain chimeric protein producing cells, which were G418 resistant cells.

[0072]

The chimeric protein producing cells obtained as described above were cultured in an F175 cell

culture flask containing D-MEM medium containing G418 (800 µg/ml) and 10% fetal bovine serum until about 60% confluent. Then, the medium was exchanged with a serum-free medium, ASF104 medium (produced by Ajinomoto), containing G418 (800 µg/ml), and the culture supernatant was collected four days later.
[0073]

The collected culture supernatant was centrifuged to remove the solid, and then 160 ml of the supernatant was passed through a Protein A Hitrap (1 ml, produced by Pharmacia) column washed with 20 mM phosphate buffer (pH 7.0) so that the chimeric protein should be adsorbed on the column. The column was sufficiently washed with 20 mM phosphate buffer (pH 7.0), and then eluted with 0.1 M citrate buffer (pH 4.5). The elution of the chimeric protein was performed with detection by a UV monitor at 280 nm, and chimeric protein eluted fractions were immediately neutralized by adding 1 M Tris-HCl buffer (pH 8.5). As a result of SDS electrophoresis, the chimeric protein obtained as described above was found to be a protein having a molecular weight of about 80 kDa as a reduced form and a molecular weight about twice as much as that of the reduced form as non-reduced form.
[0074]
[Example 4] Detection of binding of chimeric protein to immobilized mixture of von Willebrand factor and botrocetin
<1> Detection of binding of chimeric protein by ELISA using anti-mouse IgG-Fc antibodies

Botrocetin was obtained from 1 g of lyophilized product of crude venom of *Botrops jararaca* (produced by Sigma) by purification according to the method reported by Read (M.S. Read *et al.*, *Proc. Natl. Acad. Sci. USA.*, 75, 4514-4518, 1978).

[0075]

      Immobilization of a mixed solution of von Willebrand factor and botrocetin on a 96-well multititer plate was attained as follows. First, a physiological saline solution of von Willebrand factor (250 µg/ml) and a physiological saline solution of botrocetin (500 µg/ml), which were prepared in a conventional manner, were appropriately diluted, and mixed at the concentration ratios shown in Fig. 2. Then, 50 µl of each mixture was added to each well of a 96-well multititer plate (Maxisorp, produced by Nunc). The plate was left stand overnight at 4°C, and then each well was washed once with a physiological Tris buffer (150 µl, 20 mM Tris-HCl (pH 7.4), 0.15 M sodium chloride; Tris buffered saline, referred to as "TBS" hereinafter). Then, each well was added with 100 µl of TBS containing 10% BSA (bovine serum albumin), left stand for about 3 hours, and washed 3 times with TBS to obtain a von Willebrand factor immobilized plate.

[0076]

      Each well of the plate on which von Willebrand factor was immobilized in the presence of botrocetin as described above was added with 25 µl of TBS containing 1% BSA and 25 µl of a solution prepared by diluting 8 times the culture supernatant of the chimeric protein (GPIb-mIgG1Fc) producing cells obtained by using the serum free medium with TBS containing 1% BSA, incubated at room temperature for 1 hour, and washed 3 times with TBS (150 µl) containing 0.05% Tween-20. Anti-mouse IgG-Fc goat polyclonal antibodies (Catalog No. 55482, produced by Organon Teknika) were biotinylated by using Biotin Labeling Kit (Catalog No. 1418165, produced by Boehringer Mannheim) according to the protocol

attached to the kit. 50 µl of 0.1% BSA/TBA solution containing about 2 µg/ml of the above biotinylated anti-mouse IgGFc antibodies was added to each well of the plate, and incubated at room temperature for 1 hour. Further, each well was washed 3 times with TBS (150 µl) containing 0.05% Tween-20, added with 50 µl of a solution of the reagent (mixture of biotinylated alkaline phosphatase and streptavidin) contained in VECTASTAIN ABC kit (kit for biotin detection, Alkaline phosphatase standard, Catalog No. AK-5000, produced by Vector Laboratories), which solution was prepared in 0.1% BSA/TBS at 1/5 concentration of that used in the method specified in the manual, and incubated at room temperature for 1 hour. Each well was washed 5 times with TBS (150 µl) containing 0.05% Tween-20, and added with 100 µl of 100 mM $NaHCO_3$ solution containing 10 mM $MgCl_2$, in which p-nitrophenylphosphate was dissolved at 1 mg/ml, to carry out the color development reaction for about 1 hour. After the color development, absorbance at 405 nm was measured. As shown in Fig. 3, the binding of the chimeric protein was observed in a botrocetin and von Willebrand factor amount dependent manner.

[0077]

<2> Detection of chimeric protein binding using europium (Eu) labeling method

The chimeric protein (GPIb-mIgG2aFc) solution purified by the Protein A column, which was obtained in Example 3, was dialyzed against physiological saline. The solution of about 200 µg/1.5 ml was concentrated to 780 µl (concentration of about 250 µg/ml) by ultrafiltration using Centricon-10 (produced by Amicon). 500 µl of the concentrated solution (containing about 125 µg of GPIb-mIgG2aFc) was added with 50 µl of 0.5 M $NaHCO_3$, then added

with 50 µl of a solution obtained by dissolving 0.2
mg of Eu-Labeling Reagent (europium DTTA-
isothiocyanate as compound, DELFIA 1244-302,
produced by Wallac) in 250 µl of physiological
saline, and stirred at room temperature for about 40
hours to allow the reaction of europium DTTA-
isothiocyanate.
[0078]

The above reaction mixture was subjected to gel
filtration using HiLoad16/60 Superdex 75pg (inner
diameter of 16 mm, length of 60 cm, produced by
Pharmacia) to separate the unreacted reagent and the
chimeric protein. The gel filtration was performed
at a flow rate of 1 ml/minute by using physiological
saline as the eluant. The chimeric protein labeled
with Eu was recovered in fractions of the elution
volume of 40 to 48 ml. The protein was quantified
by using a protein assay kit (Protein Assay,
produced by Bio-Rad) and IgG as a standard substance.
As a result, the concentration of the labeled
chimeric protein in the eluted solution had a
concentration of 6.4 µg/ml. Hereafter, the
following experiments were conducted by using this
value as the chimeric protein concentration.
[0079]

The binding of the europium (Eu) labeled
chimeric protein and the von Willebrand factor
immobilized in the presence of botrocetin prepared
as described above was detected as follows.
According to the method mentioned in Example 4 <1>,
a mixed solution (TBS) containing 2.5 µg/ml of von
Willebrand factor and 2.5 µg/ml of botrocetin was
added to each well of a 96-well multititer plate
(microtitration plate DELFIA, 1244-550, produced by
Pharmacia Biotech), immobilized overnight and
subjected to washing, blocking and washing to

JP10-113962

prepare a von Willebrand factor immobilized plate.
[0080]

Each well of the above plate was added with 25
µl of an assay buffer containing 0.5% BSA (Assay
Buffer, Wallac DELFIA 1244-106, produced by
Pharmacia Biotech, Composition: 0.5% BSA, 0.05%
bovine γ-globulin, 0.01% Tween-40, 20 µM DTPA
(diethylenetriamine tetraacetic acid), 50 mM Tris-
HCl buffered saline (pH 7.8), 0.05% sodium azide) or
the recombinant AS1051 (in which Cys81 was replaced
with Ala, N. Fukuchiet al., WO 95/08573) at a final
concentration of 20 µg/ml as the binding inhibition
substance, further added with 25 µl of a solution of
europium (Eu) labeled chimeric protein in the same
assay buffer (100 ng/ml), shaken for 1 minute for
stirring, and then left stand at room temperature
for 2 hours. Each well of the plate was washed 5
times with TBS (150 µl) containing 0.05% Tween-20,
then added with 100 µl of a fluorescence enhancement
buffer (Enhancement buffer, 1244-104, produced by
Pharmacia Biotech, Composition: 15 µM β-NTA (2-
naphthoyltrifluoroacetone), 50 µM TOPO (tri-n-
octylphosphine oxide), 1 g/L Triton X-100, 100 mM
acetic acid/potassium hydrogen phthalate buffer),
and shaken for 1 minute for stirring. Then, the
amount of europium (Eu) was measured by using a
DELFIA Research fluorophotometer (1230 ARCUS
Fluorometer, produced by LKB). The measured values
(with addition or no addition of the binding
inhibition substance) and CV value (deviation, %)
are shown in Table 1.
[0081]
[Table 1] Measured value and CV value (%) by Eu-
labeling method

| Average value of count in control (n=80) | 26668 cpm |

40

| CV value (%) | 6.75% |
|---|---|
| Average value of count with addition of AS1051 (10 µg/ml) (n=6) | 935 cpm |
| S/N ratio | 28.5 |

[0082]
[Example 5] Detection of inhibition by binding inhibition substance for binding of von Willebrand factor and chimeric protein
<1> Detection of inhibition for binding of chimeric protein by ELISA using anti-mouse IgG-Fc antibodies

The detection was carried out in the same manner as that of Example 4 <1> except that von Willebrand factor was immobilized by using a mixed solution (TBS) containing 2.5 µg/ml of von Willebrand factor and 2.5 µg/ml of botrocetin, and a binding inhibition substance of which inhibitory activity was desired to be measured was added to the reaction of the immobilized von Willebrand factor and the culture supernatant of the chimeric protein producing cells.

[0083]
As the binding inhibition substance, AJvW-2, which is an anti-human von Willebrand factor monoclonal antibody, and a human glycoprotein Ib binding peptide derived form snake venom of *Crotalus horridus horridus* were used.

[0084]
The hybridoma producing AJvW-2 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (postal code: 305, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) on August 24, 1994, and given an accession number of FERM P-14487. Then, it was transferred to an international deposit under the provisions of the Budapest Treaty on September

29, 1995, and given an accession number of FERM BP-5248 (refer to WO96/17078). AJvW-2 can be obtained by culturing this hybridoma.

[0085]

The aforementioned human glycoprotein Ib binding peptide corresponded to a single chain peptide obtained from a multi-mer peptide derived from snake venom of *Crotalus horridus horridus* (AS1051) in which 81-cysteine residue was replaced with an alanine residue (variant type AS1051). The variant type AS1051 was obtained by modifying the gene coding for AS1051 so that the 81-cysteine residue should be replaced with an alanine residue, and expressing it in *Escherichia coli*. *E. coli* HB101/pCHA1 (*E. coli* AJ13023) harboring pCHA1 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (postal code: 305, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) on August 12, 1994 as an international deposit under the provisions of the Budapest Treaty, and given an accession number of FERM BP-4781 (refer to WO95/08573). AS1051 itself is also a human glycoprotein Ib binding peptide, and it can be detected in the same manner as that for the variant type AS1051.

[0086]

The inhibitory activities of AJvW-2 and the variant type AS1051 for the binding of the chimeric protein (*i.e.*, glycoprotein Ib) are shown in Fig. 4.

[0087]

<2> Detection of inhibition for binding of chimeric protein by europium (Eu) labeling method

The detection was carried out in the same manner as that of Example 4 <2> except that von Willebrand factor was immobilized by using a mixed

solution (TBS) containing 2.5 µg/ml of von
Willebrand factor and 2.5 µg/ml of botrocetin, and a
binding inhibition substance of which inhibitory
activity was desired to be measured was added to the
reaction of the immobilized von Willebrand factor
and the chimeric protein labeled with europium (Eu).
[0088]

As the binding inhibition substance, AJvW-2,
which is an anti-human von Willebrand factor
monoclonal antibody, and the variant type AS1051.
The inhibitory activities of the both substances for
the binding of the chimeric protein (i.e.,
glycoprotein Ib) are shown in Fig. 5.
[0089]

[Example 6] Detection of glycocalicin in plasma
<1> Detection of glycocalicin by ELISA using anti-
mouse IgG-Fc antibodies

Human plasma was prepared by collecting blood
form healthy volunteers using an injection needle of
18G, adding 1/10 volume of 3.8% aqueous sodium
citrate solution to the blood, and centrifuging the
mixture at 3000 x rpm for 10 minutes to separate a
supernatant.
[0090]

Each human plasma collected independently from
three volunteers was successively diluted 2-fold (8
times of dilution in total), and 25 µl of the plasma
was added to each well of the plate. Each well of
the plate was further added with 25 µl of a solution
prepared by diluting 8-fold a culture supernatant
obtained from culture of the chimeric protein
producing cells in a serum-free medium with TBS
containing 0.1% BSA, and incubated at room
temperature for 1 hour. The subsequent reactions
and color development were performed in the same
manner as Example 5 <1>, and the average values of

43

the results are shown in Fig. 6.
[0091]

The blood concentration of glycocalicin in healthy people was reported to be about 2 μg/ml. On the other hand, the glycocalicin concentration showing 50% binding inhibition in this detection system was about 400 ng/ml. From this fact, it was considered that a glycocalicin amount of 60 ng/ml or more could sufficiently be measured in view of the linearity of the plot.
[0092]
<2> Detection of glycocalicin using chimeric protein labeled with europium (Eu)

Each human plasma independently prepared in the same manner as the above <1> were successively diluted 2-fold with TBS (8 times of dilution in total), and 25 μl each of the diluted plasma was added to each well of a von Willebrand factor immobilized plate prepared in the same manner as in Example 5 <1> (microtitration plate DELFIA, 1244-550, produced by Pharmacia Biotech, was used as the base plate). Further, 25 μl a solution of the chimeric protein labeled with europium (Eu) in assay buffer prepared in the same manner as in Example 4 <1> (100 ng/ml, Assay Buffer; 1244-106, produced by Pharmacia Biotech) was added to each well of the plate for reaction. The subsequent washing and measurement were performed in the same manner as in Example 5 <2>, and the average values of the results are shown in Fig. 7.
[0093]

The blood concentration of glycocalicin in healthy people was reported to be about 2 μg/ml. On the other hand, the glycocalicin concentration showing 50% binding inhibition was about 60 ng/ml in this detection system. From this fact, it was

44

considered that a glycocalicin amount of 30 ng/ml or
more could sufficiently be measured.
[0094]
[Advantageous effect of the invention]
    According to the present invention, the binding
of glycoprotein Ib and von Willebrand factor or
inhibition thereof can be detected in a simple
manner.  According to the method of the present
invention, there are provided a simple method for
quantification of glycocalicin with superior
quantification ability, and a simple method for
measurement of a substance inhibiting the binding of
von Willebrand factor and glycoprotein Ib with
superior operability.
[0095]

    If von Willebrand factor is immobilized in the
presence of a binding inducing substance such as
botrocetin, the binding of von Willebrand factor and
glycoprotein Ib can be observed in a simple manner
with good reproducibility, without adding a binding
inducing substance such as botrocetin or ristocetin
to a liquid phase.
[0096]

    Further, by utilizing the chimeric protein of
the present invention, it becomes unnecessary to
prepare or obtain monoclonal antibodies for
detection or quantification of a binding inhibition
substance such as glycocalicin.
    Moreover, the present invention also provides a
method for preparing a chimeric molecule (chimeric
protein) that comprises a partial protein of
glycoprotein Ib bound to the Fc region of
immunoglobulin molecule by using animal cells.

[0097]
[Sequence Listing]
SEQUENCE ID NO: 1
Length: 36
Type: nucleic acid
Number of chain: 1
Topology: linear
Type of sequence: other nucleic acid, Artificial DNA
Sequence:
atctgtgagg tctccaaagt ggccagccac ctagaa                    36

[0098]
SEQUENCE ID NO: 2
Length: 29
Type: nucleic acid
Number of chain: 1
Topology: linear
Type of sequence: other nucleic acid, Artificial DNA
Sequence:
atatctagat gtgcccaggg attgtggtt                    29

[0099]
SEQUENCE ID NO: 3
Length: 36
Type: nucleic acid
Number of chain: 1
Topology: linear
Type of sequence: other nucleic acid, Artificial DNA
Sequence:
ataaagcttc tcgagtcatt taccaggaga gtggga                    36

[0100]
SEQUENCE ID NO: 4
Length: 684
Type: nucleic acid
Number of chain: 2
Topology: linear
Type of sequence: cDNA to mRNA

Sequence:

```
gtg ccc agg gat tgt ggt tgt aag cct tgc ata tgt aca gtc cca gaa      48
Val Pro Arg Asp Cys Gly Cys Lys Pro Cys Ile Cys Thr Val Pro Glu
 1               5                   10                  15

gta tca tct gtc ttc atc ttc ccc cca aag ccc aag gat gtg ctc acc      96
Val Ser Ser Val Phe Ile Phe Pro Pro Lys Pro Lys Asp Val Leu Thr
            20                  25                  30

att act ctg act cct aag gtc acg tgt gtt gtg gta gac atc agc aag     144
Ile Thr Leu Thr Pro Lys Val Thr Cys Val Val Val Asp Ile Ser Lys
        35                  40                  45

gat gat ccc gag gtc cag ttc agc tgg ttt gta gat gat gtg gag gtg     192
Asp Asp Pro Glu Val Gln Phe Ser Trp Phe Val Asp Asp Val Glu Val
    50                  55                  60

cac aca gct cag acg caa ccc cgg gag gag cag ttc aac agc act ttc     240
His Thr Ala Gln Thr Gln Pro Arg Glu Glu Gln Phe Asn Ser Thr Phe
65                  70                  75                  80

cgc tca gtc agt gaa ctt ccc atc atg cac cag gac tgg ctc aat ggc     288
Arg Ser Val Ser Glu Leu Pro Ile Met His Gln Asp Trp Leu Asn Gly
                85                  90                  95

aag gag ttc aaa tgc agg gta aac agt gca gct ttc cct gcc ccc atc     336
Lys Glu Phe Lys Cys Arg Val Asn Ser Ala Ala Phe Pro Ala Pro Ile
            100                 105                 110

gag aaa acc atc tcc aaa acc aaa ggc aga ccg aag gct cca cag gtg     384
Glu Lys Thr Ile Ser Lys Thr Lys Gly Arg Pro Lys Ala Pro Gln Val
            115                 120                 125

tac acc att cca cct ccc aag gag cag atg gcc aag gat aaa gtc agt     432
Tyr Thr Ile Pro Pro Pro Lys Glu Gln Met Ala Lys Asp Lys Val Ser
    130                 135                 140

ctg acc tgc atg ata aca gac ttc ttc cct gaa gac att act gtg gag     480
Leu Thr Cys Met Ile Thr Asp Phe Phe Pro Glu Asp Ile Thr Val Glu
145                 150                 155                 160

tgg cag tgg aat ggg cag cca gcg gag aac tac aag aac act cag ccc     528
Trp Gln Trp Asn Gly Gln Pro Ala Glu Asn Tyr Lys Asn Thr Gln Pro
                165                 170                 175

atc atg gac aca gat ggc tct tac ttc gtc tac agc aag ctc aat gtg     576
Ile Met Asp Thr Asp Gly Ser Tyr Phe Val Tyr Ser Lys Leu Asn Val
                180                 185                 190

cag aag agc aac tgg gag gca gga aat act ttc acc tgc tct gtg tta     624
Gln Lys Ser Asn Trp Glu Ala Gly Asn Thr Phe Thr Cys Ser Val Leu
            195                 200                 205

cat gag ggc ctg cac aac cac cat act gag aag agc ctc tcc cac tct     672
His Glu Gly Leu His Asn His His Thr Glu Lys Ser Leu Ser His Ser
    210                 215                 220
```

cct ggt aaa tga                                                                    684
Pro Gly Lys
225


[0101]

SEQUENCE ID NO: 5

Length: 227

Type: amino acid

Topology: linear

Type of sequence: peptide

Sequence:

Val Pro Arg Asp Cys Gly Cys Lys Pro Cys Ile Cys Thr Val Pro Glu
 1             5                 10                 15
Val Ser Ser Val Phe Ile Phe Pro Pro Lys Pro Lys Asp Val Leu Thr
             20                 25                 30
Ile Thr Leu Thr Pro Lys Val Thr Cys Val Val Val Asp Ile Ser Lys
         35                 40                 45
Asp Asp Pro Glu Val Gln Phe Ser Trp Phe Val Asp Asp Val Glu Val
     50                 55                 60
His Thr Ala Gln Thr Gln Pro Arg Glu Glu Gln Phe Asn Ser Thr Phe
 65             70                 75                 80
Arg Ser Val Ser Glu Leu Pro Ile Met His Gln Asp Trp Leu Asn Gly
                 85                 90                 95
Lys Glu Phe Lys Cys Arg Val Asn Ser Ala Ala Phe Pro Ala Pro Ile
             100                105                110
Glu Lys Thr Ile Ser Lys Thr Lys Gly Arg Pro Lys Ala Pro Gln Val
             115                120                125
Tyr Thr Ile Pro Pro Pro Lys Glu Gln Met Ala Lys Asp Lys Val Ser
     130                135                140
Leu Thr Cys Met Ile Thr Asp Phe Phe Pro Glu Asp Ile Thr Val Glu
145                150                155                160
Trp Gln Trp Asn Gly Gln Pro Ala Glu Asn Tyr Lys Asn Thr Gln Pro
                 165                170                175
Ile Met Asp Thr Asp Gly Ser Tyr Phe Val Tyr Ser Lys Leu Asn Val
             180                185                190
Gln Lys Ser Asn Trp Glu Ala Gly Asn Thr Phe Thr Cys Ser Val Leu
             195                200                205
His Glu Gly Leu His Asn His His Thr Glu Lys Ser Leu Ser His Ser
     210                215                220
Pro Gly Lys
225

48

[0102]
SEQUENCE ID NO: 6
Length: 1689
Type: nucleic acid
Number of chain: 2
Topology: linear
Type of sequence: other nucleic acid, Fusion DNA
Feature:
　　　Name/key: CDS
　　　Location: 1..1689
　　　Method for determining the feature: S

　　　Name/key: sig_peptide
　　　Location: 1..48
　　　Method for determining the feature: S
　　　Name/Key: mat_peptide
　　　Location: 49..1689
　　　Method for determining the feature: S
Sequence:
```
atg cct ctc ctc ctc ttg ctg ctc ctg ctg cca agc ccc tta cac ccc   48
Met Pro Leu Leu Leu Leu Leu Leu Leu Leu Pro Ser Pro Leu His Pro
-16 -15               -10                   -5
cac ccc atc tgt gag gtc tcc aaa gtg gcc agc cac cta gaa gtg aac   96
His Pro Ile Cys Glu Val Ser Lys Val Ala Ser His Leu Glu Val Asn
 1               5                   10                   15
tgt gac aag agg aat ctg aca gcg ctg cct cca gac ctg ccg aaa gac  144
Cys Asp Lys Arg Asn Leu Thr Ala Leu Pro Pro Asp Leu Pro Lys Asp
            20                   25                   30
aca acc atc ctc cac ctg agt gag aac ctc ctg tac acc ttc tcc ctg  192
Thr Thr Ile Leu His Leu Ser Glu Asn Leu Leu Tyr Thr Phe Ser Leu
            35                   40                   45
gca acc ctg atg cct tac act cgc ctc act cag ctg aac cta gat agg  240
Ala Thr Leu Met Pro Tyr Thr Arg Leu Thr Gln Leu Asn Leu Asp Arg
      50                   55                   60
tgc gag ctc acc aag ctc cag gtc gat ggg acg ctg cca gtg ctg ggg  288
Cys Glu Leu Thr Lys Leu Gln Val Asp Gly Thr Leu Pro Val Leu Gly
 65                   70                   75                   80
acc ctg gat cta tcc cac aat cag ctg caa agc ctg ccc ttg cta ggg  336
Thr Leu Asp Leu Ser His Asn Gln Leu Gln Ser Leu Pro Leu Leu Gly
                  85                   90                   95
```

```
cag aca ctg cct gct ctc acc gtc ctg gac gtc tcc ttc aac cgg ctg     384
Gln Thr Leu Pro Ala Leu Thr Val Leu Asp Val Ser Phe Asn Arg Leu
            100             105             110
acc tcg ctg cct ctt ggt gcc ctg cgt ggt ctt ggc gaa ctc caa gag     432
Thr Ser Leu Pro Leu Gly Ala Leu Arg Gly Leu Gly Glu Leu Gln Glu
            115             120             125
ctc tac ctg aaa ggc aat gag ctg aag acc ctg ccc cca ggg ctc ctg     480
Leu Tyr Leu Lys Gly Asn Glu Leu Lys Thr Leu Pro Pro Gly Leu Leu
            130             135             140
acg ccc aca ccc aag ctg gag aag ctc agt ctg gct aac aac aac ttg     528
Thr Pro Thr Pro Lys Leu Glu Lys Leu Ser Leu Ala Asn Asn Asn Leu
145             150             155             160
act gag ctc ccc gct ggg ctc ctg aat ggg ctg gag aat ctc gac acc     576
Thr Glu Leu Pro Ala Gly Leu Leu Asn Gly Leu Glu Asn Leu Asp Thr
            165             170             175
ctt ctc ctc caa gag aac tcg ctg tat aca ata cca aag ggc ttt ttt     624
Leu Leu Leu Gln Glu Asn Ser Leu Tyr Thr Ile Pro Lys Gly Phe Phe
            180             185             190
ggg tcc cac ctc ctg cct ttt gct ttt ctc cac ggg aac ccc tgg tta     672
Gly Ser His Leu Leu Pro Phe Ala Phe Leu His Gly Asn Pro Trp Leu
            195             200             205
tgc aac tgt gag atc ctc tat ttt cgt cgc tgg ctg cag gac aat gct     720
Cys Asn Cys Glu Ile Leu Tyr Phe Arg Arg Trp Leu Gln Asp Asn Ala
            210             215             220
gaa aat gtc tac gta tgg aag caa ggt gtg gac gtc aag gcc atg acc     768
Glu Asn Val Tyr Val Trp Lys Gln Gly Val Asp Val Lys Ala Met Thr
225             230             235             240
tct aac gtg gcc agt gtg cag tgt gac aat tca gac aag ttt ccc gtc     816
Ser Asn Val Ala Ser Val Gln Cys Asp Asn Ser Asp Lys Phe Pro Val
            245             250             255
tac aaa tac cca gga aag ggg tgc ccc acc ctt ggt gat gaa ggt gac     864
Tyr Lys Tyr Pro Gly Lys Gly Cys Pro Thr Leu Gly Asp Glu Gly Asp
            260             265             270
aca gac cta tat gat tac tac cca gaa gag gac act gag ggc gat aag     912
Thr Asp Leu Tyr Asp Tyr Tyr Pro Glu Glu Asp Thr Glu Gly Asp Lys
            275             280             285
gtg cgt gcc aca agg act gtg gtc aag ttc ccc acc aaa gcc cat aca     960
Val Arg Ala Thr Arg Thr Val Val Lys Phe Pro Thr Lys Ala His Thr
            290             295             300
acc ccc tgg ggt cta ttc tac tca tgg tcc act gct tct cta gac gtg    1008
Thr Pro Trp Gly Leu Phe Tyr Ser Trp Ser Thr Ala Ser Leu Asp Val
305             310             315             320
ccc agg gat tgt ggt tgt aag cct tgc ata tgt aca gtc cca gaa gta    1056
```

```
Pro Arg Asp Cys Gly Cys Lys Pro Cys Ile Cys Thr Val Pro Glu Val
            325             330             335
tca tct gtc ttc atc ttc ccc cca aag ccc aag gat gtg ctc acc att   1104
Ser Ser Val Phe Ile Phe Pro Pro Lys Pro Lys Asp Val Leu Thr Ile
            340             345             350
act ctg act cct aag gtc acg tgt gtt gtg gta gac atc agc aag gat   1152
Thr Leu Thr Pro Lys Val Thr Cys Val Val Val Asp Ile Ser Lys Asp
            355             360             365
gat ccc gag gtc cag ttc agc tgg ttt gta gat gat gtg gag gtg cac   1200
Asp Pro Glu Val Gln Phe Ser Trp Phe Val Asp Asp Val Glu Val His
            370             375             380
aca gct cag acg caa ccc cgg gag gag cag ttc aac agc act ttc cgc   1248
Thr Ala Gln Thr Gln Pro Arg Glu Glu Gln Phe Asn Ser Thr Phe Arg
385             390             395             400
tca gtc agt gaa ctt ccc atc atg cac cag gac tgg ctc aat ggc aag   1296
Ser Val Ser Glu Leu Pro Ile Met His Gln Asp Trp Leu Asn Gly Lys
            405             410             415
gag ttc aaa tgc agg gta aac agt gca gct ttc cct gcc ccc atc gag   1344
Glu Phe Lys Cys Arg Val Asn Ser Ala Ala Phe Pro Ala Pro Ile Glu
            420             425             430
aaa acc atc tcc aaa acc aaa ggc aga ccg aag gct cca cag gtg tac   1392
Lys Thr Ile Ser Lys Thr Lys Gly Arg Pro Lys Ala Pro Gln Val Tyr
            435             440             445
acc att cca cct ccc aag gag cag atg gcc aag gat aaa gtc agt ctg   1440
Thr Ile Pro Pro Pro Lys Glu Gln Met Ala Lys Asp Lys Val Ser Leu
            450             455             460
acc tgc atg ata aca gac ttc ttc cct gaa gac att act gtg gag tgg   1488
Thr Cys Met Ile Thr Asp Phe Phe Pro Glu Asp Ile Thr Val Glu Trp
465             470             ·475            480
cag tgg aat ggg cag cca gcg gag aac tac aag aac act cag ccc atc   1536
Gln Trp Asn Gly Gln Pro Ala Glu Asn Tyr Lys Asn Thr Gln Pro Ile
            485             490             495
atg gac aca gat ggc tct tac ttc gtc tac agc aag ctc aat gtg cag   1584
Met Asp Thr Asp Gly Ser Tyr Phe Val Tyr Ser Lys Leu Asn Val Gln
            500             505             510
aag agc aac tgg gag gca gga aat act ttc acc tgc tct gtg tta cat   1632
Lys Ser Asn Trp Glu Ala Gly Asn Thr Phe Thr Cys Ser Val Leu His
            515             520             525
gag ggc ctg cac aac cac cat act gag aag agc ctc tcc cac tct cct   1680
Glu Gly Leu His Asn His His Thr Glu Lys Ser Leu Ser His Ser Pro
            530             535             540
ggt aaa tga                                                        1689
Gly Lys
```

545

[0103]
SEQUENCE ID NO: 7
Length: 562
Type: amino acid
Topology: linear
Type of sequence: peptide
Sequence:

```
Met Pro Leu Leu Leu Leu Leu Leu Leu Leu Pro Ser Pro Leu His Pro
-16 -15              -10               -5
His Pro Ile Cys Glu Val Ser Lys Val Ala Ser His Leu Glu Val Asn
    1           5               10                  15
Cys Asp Lys Arg Asn Leu Thr Ala Leu Pro Pro Asp Leu Pro Lys Asp
            20              25          ·       30
Thr Thr Ile Leu His Leu Ser Glu Asn Leu Leu Tyr Thr Phe Ser Leu
        35              40                  45
Ala Thr Leu Met Pro Tyr Thr Arg Leu Thr Gln Leu Asn Leu Asp Arg
    ·  50              55                  60
Cys Glu Leu Thr Lys Leu Gln Val Asp Gly Thr Leu Pro Val Leu Gly
65              70              75                  80
Thr Leu Asp Leu Ser His Asn Gln Leu Gln Ser Leu Pro Leu Leu Gly
                85              90                  95
Gln Thr Leu Pro Ala Leu Thr Val Leu Asp Val Ser Phe Asn Arg Leu
            100             105             110
Thr Ser Leu Pro Leu Gly Ala Leu Arg Gly Leu Gly Glu Leu Gln Glu
        115             120             125
Leu Tyr Leu Lys Gly Asn Glu Leu Lys Thr Leu Pro Pro Gly Leu Leu
    130             135             140
Thr Pro Thr Pro Lys Leu Glu Lys Leu Ser Leu Ala Asn Asn Asn Leu
145             150             155             160
Thr Glu Leu Pro Ala Gly Leu Leu Asn Gly Leu Glu Asn Leu Asp Thr
            165             170             175
Leu Leu Leu Gln Glu Asn Ser Leu Tyr Thr Ile Pro Lys Gly Phe Phe
            180             185             190
Gly Ser His Leu Leu Pro Phe Ala Phe Leu His Gly Asn Pro Trp Leu
        195             200             205
Cys Asn Cys Glu Ile Leu Tyr Phe Arg Arg Trp Leu Gln Asp Asn Ala
        210             215             220
Glu Asn Val Tyr Val Trp Lys Gln Gly Val Asp Val Lys Ala Met Thr
225             230             235                 240
Ser Asn Val Ala Ser Val Gln Cys Asp Asn Ser Asp Lys Phe Pro Val
```

```
                     245                      250                      255
Tyr Lys Tyr Pro Gly Lys Gly Cys Pro Thr Leu Gly Asp Glu Gly Asp
                260                      265                      270
Thr Asp Leu Tyr Asp Tyr Tyr Pro Glu Glu Asp Thr Glu Gly Asp Lys
            275                      280                      285
Val Arg Ala Thr Arg Thr Val Val Lys Phe Pro Thr Lys Ala His Thr
        290                      295                      300
Thr Pro Trp Gly Leu Phe Tyr Ser Trp Ser Thr Ala Ser Leu Asp Val
305                      310                      315                      320
Pro Arg Asp Cys Gly Cys Lys Pro Cys Ile Cys Thr Val Pro Glu Val
                325                      330                      335
Ser Ser Val Phe Ile Phe Pro Pro Lys Pro Lys Asp Val Leu Thr Ile
            340                      345                      350
Thr Leu Thr Pro Lys Val Thr Cys Val Val Val Asp Ile Ser Lys Asp
            355                      360                      365
Asp Pro Glu Val Gln Phe Ser Trp Phe Val Asp Asp Val Glu Val His
        370                      375                      380
Thr Ala Gln Thr Gln Pro Arg Glu Glu Gln Phe Asn Ser Thr Phe Arg
385                      390                      395                      400
Ser Val Ser Glu Leu Pro Ile Met His Gln Asp Trp Leu Asn Gly Lys
                405                      410                      415
Glu Phe Lys Cys Arg Val Asn Ser Ala Ala Phe Pro Ala Pro Ile Glu
                420                      425                      430
Lys Thr Ile Ser Lys Thr Lys Gly Arg Pro Lys Ala Pro Gln Val Tyr
            435                      440                      445
Thr Ile Pro Pro Pro Lys Glu Gln Met Ala Lys Asp Lys Val Ser Leu
            450                      455                      460
Thr Cys Met Ile Thr Asp Phe Phe Pro Glu Asp Ile Thr Val Glu Trp
465                      470                      475                      480
Gln Trp Asn Gly Gln Pro Ala Glu Asn Tyr Lys Asn Thr Gln Pro Ile
                485                      490                      495
Met Asp Thr Asp Gly Ser Tyr Phe Val Tyr Ser Lys Leu Asn Val Gln
                500                      505                      510
Lys Ser Asn Trp Glu Ala Gly Asn Thr Phe Thr Cys Ser Val Leu His
            515                      520                      525
Glu Gly Leu His Asn His His Thr Glu Lys Ser Leu Ser His Ser Pro
            530                      535                      540
Gly Lys
545
```

[0104]
SEQUENCE ID NO: 8
Length: 30

Type: nucleic acid

Number of chain: 1

Topology: linear

Type of sequence: other nucleic acid, Artificial DNA

Sequence:

agctaggatc cgagcccaga gggcccacaa                                    30


[0105]

SEQUENCE ID NO: 9

Length: 44

Type: nucleic acid

Number of chain: 1

Topology: linear

Type of sequence: other nucleic acid, Artificial DNA

Sequence:

cccaagcttc tcgagacata cctttcattt acccggagtc cggga                   44


[0106]

SEQUENCE ID NO: 10

Length: 729

Type: nucleic acid

Number of chain: 2

Topology: linear

Type of sequence: cDNA to mRNA

Origine:

      Organism: mouse

      Type of cell: hybridoma cell strain W6/32

Feature:

      Name/key: CDS

      Location: 7..708

      Method for determining the feature: S

Sequence:

ggatcc gag ccc aga ggg ccc aca atc aag ccc tgt cct cca tgc aaa      48
      Glu Pro Arg Gly Pro Thr Ile Lys Pro Cys Pro Pro Cys Lys
      1          5               10

tgc cca gca cct aac ctc ttg ggt gga cca tcc gtc ttc atc ttc cct     96
Cys Pro Ala Pro Asn Leu Leu Gly Gly Pro Ser Val Phe Ile Phe Pro
15            20            25            30

```
cca aag atc aag gat gta ctc atg atc tcc ctg agc ccc ata gtc aca    144
Pro Lys Ile Lys Asp Val Leu Met Ile Ser Leu Ser Pro Ile Val Thr
             35                  40                  45

tgt gtg gtg gtg gat gtg agc gag gat gac cca gat gtc cag atc agc    192
Cys Val Val Val Asp Val Ser Glu Asp Asp Pro Asp Val Gln Ile Ser
             50                  55                  60

tgg ttt gtg aac aac gtg gaa gta cac aca gct cag aca caa acc cat    240
Trp Phe Val Asn Asn Val Glu Val His Thr Ala Gln Thr Gln Thr His
             65                  70                  75

aga gag gat tac aac agt act ctc cgg gtg gtc agt gcc ctc ccc atc    288
Arg Glu Asp Tyr Asn Ser Thr Leu Arg Val Val Ser Ala Leu Pro Ile
         80                  85                  90

cag cac cag gac tgg atg agt ggc aag gag ttc aaa tgc aag gtc aac    336
Gln His Gln Asp Trp Met Ser Gly Lys Glu Phe Lys Cys Lys Val Asn
     95                  100                 105                 110

aac aaa gac ctg cca gcg ccc atc gag aga acc atc tca aaa ccc aaa    384
Asn Lys Asp Leu Pro Ala Pro Ile Glu Arg Thr Ile Ser Lys Pro Lys
             115                 120                 125

ggg tca gta aga gct cca cag gta tat gtc ttg cct cca cca gaa gaa    432
Gly Ser Val Arg Ala Pro Gln Val Tyr Val Leu Pro Pro Pro Glu Glu
             130                 135                 140

gag atg act aag aaa cag gtc act ctg acc tgc atg gtc aca gac ttc    480
Glu Met Thr Lys Lys Gln Val Thr Leu Thr Cys Met Val Thr Asp Phe
         145                 150                 155

atg cct gaa gac att tac gtg gag tgg acc aac aac ggg aaa aca gag    528
Met Pro Glu Asp Ile Tyr Val Glu Trp Thr Asn Asn Gly Lys Thr Glu
         160                 165                 170

cta aac tac aag aac act gaa cca gtc ctg gac tct gat ggt tct tac    576
Leu Asn Tyr Lys Asn Thr Glu Pro Val Leu Asp Ser Asp Gly Ser Tyr
175                 180                 185                 190

ttc atg tac agc aag ctg aga gtg gaa aag aag aac tgg gtg gaa aga    624
Phe Met Tyr Ser Lys Leu Arg Val Glu Lys Lys Asn Trp Val Glu Arg
             195                 200                 205

aat agc tac tcc tgt tca gtg gtc cac gag ggt ctg cac aat cac cac    672
Asn Ser Tyr Ser Cys Ser Val Val His Glu Gly Leu His Asn His His
             210                 215                 220

acg act aag agc ttc tcc cgg act ccg ggt aaa tgaaaggtat gtctcgagaa  725
Thr Thr Lys Ser Phe Ser Arg Thr Pro Gly Lys
             225                 230

gctt                                                               729
```

[0107]
SEQUENCE ID NO: 11

Length: 233

Type: amino acid

Topology: linear

Type of sequence: peptide

Sequence:

Glu Pro Arg Gly Pro Thr Ile Lys Pro Cys Pro Pro Cys Lys Cys Pro
 1             5                 10                15

Ala Pro Asn Leu Leu Gly Gly Pro Ser Val Phe Ile Phe Pro Pro Lys
             20              25              30

Ile Lys Asp Val Leu Met Ile Ser Leu Ser Pro Ile Val Thr Cys Val
         35              40              45

Val Val Asp Val Ser Glu Asp Asp Pro Asp Val Gln Ile Ser Trp Phe
     50              55              60

Val Asn Asn Val Glu Val His Thr Ala Gln Thr Gln Thr His Arg Glu
 65              70              75              80

Asp Tyr Asn Ser Thr Leu Arg Val Val Ser Ala Leu Pro Ile Gln His
             85              90              95

Gln Asp Trp Met Ser Gly Lys Glu Phe Lys Cys Lys Val Asn Asn Lys
             100             105             110

Asp Leu Pro Ala Pro Ile Glu Arg Thr Ile Ser Lys Pro Lys Gly Ser
         115             120             125

Val Arg Ala Pro Gln Val Tyr Val Leu Pro Pro Pro Glu Glu Glu Met
     130             135             140

Thr Lys Lys Gln Val Thr Leu Thr Cys Met Val Thr Asp Phe Met Pro
145             150             155             160

Glu Asp Ile Tyr Val Glu Trp Thr Asn Asn Gly Lys Thr Glu Leu Asn
             165             170             175

Tyr Lys Asn Thr Glu Pro Val Leu Asp Ser Asp Gly Ser Tyr Phe Met
             180             185             190

Tyr Ser Lys Leu Arg Val Glu Lys Lys Asn Trp Val Glu Arg Asn Ser
             195             200             205

Tyr Ser Cys Ser Val Val His Glu Gly Leu His Asn His His Thr Thr
     210             215             220

Lys Ser Phe Ser Arg Thr Pro Gly Lys
225             230

[0108]

SEQUENCE ID NO: 12

Length: 30

Type: nucleic acid

Number of chain: 1

Topology: linear

Type of sequence: other nucleic acid, Artificial DNA

Sequence:

agctatctag acgagcccag agggcccaca     30

[0109]

SEQUENCE ID NO: 13

Length: 1707

Type: nucleic acid

Number of chain: 2

Topology: linear

Type of sequence: other nucleic acid, Fusion DNA

Feature:

    Name/key: CDS

    Location: 1..1707

    Method for determining the feature: S


    Name/key: sig_peptide

    Location: 1..480

    Method of determining the feature: S


    Name/key: mat_peptide

    Location: 49..1704

    Method of determining the feature: S

Sequence:

```
atg cct ctc ctc ctc ttg ctg ctc ctg ctg cca agc ccc tta cac ccc    48
Met Pro Leu Leu Leu Leu Leu Leu Leu Leu Pro Ser Pro Leu His Pro
-16 -15                 -10                 -5
cac ccc atc tgt gag gtc tcc aaa gtg gcc agc cac cta gaa gtg aac    96
His Pro Ile Cys Glu Val Ser Lys Val Ala Ser His Leu Glu Val Asn
  1           5                 10                15
tgt gac aag agg aat ctg aca gcg ctg cct cca gac ctg ccg aaa gac   144
Cys Asp Lys Arg Asn Leu Thr Ala Leu Pro Pro Asp Leu Pro Lys Asp
            20                25                30
aca acc atc ctc cac ctg agt gag aac ctc ctg tac acc ttc tcc ctg   192
Thr Thr Ile Leu His Leu Ser Glu Asn Leu Leu Tyr Thr Phe Ser Leu
          35                40                45
gca acc ctg atg cct tac act cgc ctc act cag ctg aac cta gat agg   240
Ala Thr Leu Met Pro Tyr Thr Arg Leu Thr Gln Leu Asn Leu Asp Arg
```

```
                50                      55                      60
tgc gag ctc acc aag ctc cag gtc gat ggg acg ctg cca gtg ctg ggg    288
Cys Glu Leu Thr Lys Leu Gln Val Asp Gly Thr Leu Pro Val Leu Gly
 65                      70                      75                      80

acc ctg gat cta tcc cac aat cag ctg caa agc ctg ccc ttg cta ggg    336
Thr Leu Asp Leu Ser His Asn Gln Leu Gln Ser Leu Pro Leu Leu Gly
                    85                      90                      95

cag aca ctg cct gct ctc acc gtc ctg gac gtc tcc ttc aac cgg ctg    384
Gln Thr Leu Pro Ala Leu Thr Val Leu Asp Val Ser Phe Asn Arg Leu
                100                     105                     110

acc tcg ctg cct ctt ggt gcc ctg cgt ggt ctt ggc gaa ctc caa gag    432
Thr Ser Leu Pro Leu Gly Ala Leu Arg Gly Leu Gly Glu Leu Gln Glu
                115                     120                     125

ctc tac ctg aaa ggc aat gag ctg aag acc ctg ccc cca ggg ctc ctg    480
Leu Tyr Leu Lys Gly Asn Glu Leu Lys Thr Leu Pro Pro Gly Leu Leu
            130                     135                     140

acg ccc aca ccc aag ctg gag aag ctc agt ctg gct aac aac aac ttg    528
Thr Pro Thr Pro Lys Leu Glu Lys Leu Ser Leu Ala Asn Asn Asn Leu
145                     150                     155                     160

act gag ctc ccc gct ggg ctc ctg aat ggg ctg gag aat ctc gac acc    576
Thr Glu Leu Pro Ala Gly Leu Leu Asn Gly Leu Glu Asn Leu Asp Thr
                165                     170                     175

ctt ctc ctc caa gag aac tcg ctg tat aca ata cca aag ggc ttt ttt    624
Leu Leu Leu Gln Glu Asn Ser Leu Tyr Thr Ile Pro Lys Gly Phe Phe
                180                     185                     190

ggg tcc cac ctc ctg cct ttt gct ttt ctc cac ggg aac ccc tgg tta    672
Gly Ser His Leu Leu Pro Phe Ala Phe Leu His Gly Asn Pro Trp Leu
            195                     200                     205

tgc aac tgt gag atc ctc tat ttt cgt cgc tgg ctg cag gac aat gct    720
Cys Asn Cys Glu Ile Leu Tyr Phe Arg Arg Trp Leu Gln Asp Asn Ala
        210                     215                     220

gaa aat gtc tac gta tgg aag caa ggt gtg gac gtc aag gcc atg acc    768
Glu Asn Val Tyr Val Trp Lys Gln Gly Val Asp Val Lys Ala Met Thr
225                     230                     235                     240

tct aac gtg gcc agt gtg cag tgt gac aat tca gac aag ttt ccc gtc    816
Ser Asn Val Ala Ser Val Gln Cys Asp Asn Ser Asp Lys Phe Pro Val
                245                     250                     255

tac aaa tac cca gga aag ggg tgc ccc acc ctt ggt gat gaa ggt gac    864
Tyr Lys Tyr Pro Gly Lys Gly Cys Pro Thr Leu Gly Asp Glu Gly Asp
            260                     265                     270

aca gac cta tat gat tac tac cca gaa gag gac act gag ggc gat aag    912
Thr Asp Leu Tyr Asp Tyr Tyr Pro Glu Glu Asp Thr Glu Gly Asp Lys
            275                     280                     285
```

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gtg cgt gcc aca agg act gtg gtc aag ttc ccc acc aaa gcc cat aca 960
Val Arg Ala Thr Arg Thr Val Val Lys Phe Pro Thr Lys Ala His Thr
    290             295             300

acc ccc tgg ggt cta ttc tac tca tgg tcc act gct tct cta gac gag 1008
Thr Pro Trp Gly Leu Phe Tyr Ser Trp Ser Thr Ala Ser Leu Asp Glu
305             310             315             320

ccc aga ggg ccc aca atc aag ccc tgt cct cca tgc aaa tgc cca gca 1056
Pro Arg Gly Pro Thr Ile Lys Pro Cys Pro Pro Cys Lys Cys Pro Ala
                325             330             335

cct aac ctc ttg ggt gga cca tcc gtc ttc atc ttc cct cca aag atc 1104
Pro Asn Leu Leu Gly Gly Pro Ser Val Phe Ile Phe Pro Pro Lys Ile
                340             345             350

aag gat gta ctc atg atc tcc ctg agc ccc ata gtc aca tgt gtg gtg 1152
Lys Asp Val Leu Met Ile Ser Leu Ser Pro Ile Val Thr Cys Val Val
                355             360             365

gtg gat gtg agc gag gat gac cca gat gtc cag atc agc tgg ttt gtg 1200
Val Asp Val Ser Glu Asp Asp Pro Asp Val Gln Ile Ser Trp Phe Val
                370             375             380

aac aac gtg gaa gta cac aca gct cag aca caa acc cat aga gag gat 1248
Asn Asn Val Glu Val His Thr Ala Gln Thr Gln Thr His Arg Glu Asp
385             390             395             400

tac aac agt act ctc cgg gtg gtc agt gcc ctc ccc atc cag cac cag 1296
Tyr Asn Ser Thr Leu Arg Val Val Ser Ala Leu Pro Ile Gln His Gln
                405             410             415

gac tgg atg agt ggc aag gag ttc aaa tgc aag gtc aac aac aaa gac 1344
Asp Trp Met Ser Gly Lys Glu Phe Lys Cys Lys Val Asn Asn Lys Asp
                420             425             430

ctg cca gcg ccc atc gag aga acc atc tca aaa ccc aaa ggg tca gta 1392
Leu Pro Ala Pro Ile Glu Arg Thr Ile Ser Lys Pro Lys Gly Ser Val
                435             440             445

aga gct cca cag gta tat gtc ttg cct cca cca gaa gaa gag atg act 1440
Arg Ala Pro Gln Val Tyr Val Leu Pro Pro Pro Glu Glu Glu Met Thr
                450             455             460

aag aaa cag gtc act ctg acc tgc atg gtc aca gac ttc atg cct gaa 1488
Lys Lys Gln Val Thr Leu Thr Cys Met Val Thr Asp Phe Met Pro Glu
465             470             475             480

gac att tac gtg gag tgg acc aac aac ggg aaa aca gag cta aac tac 1536
Asp Ile Tyr Val Glu Trp Thr Asn Asn Gly Lys Thr Glu Leu Asn Tyr
                485             490             495

aag aac act gaa cca gtc ctg gac tct gat ggt tct tac ttc atg tac 1584
Lys Asn Thr Glu Pro Val Leu Asp Ser Asp Gly Ser Tyr Phe Met Tyr
                500             505             510

agc aag ctg aga gtg gaa aag aag aac tgg gtg gaa aga aat agc tac 1632

59

```
Ser Lys Leu Arg Val Glu Lys Lys Asn Trp Val Glu Arg Asn Ser Tyr
        515                 520                 525
tcc tgt tca gtg gtc cac gag ggt ctg cac aat cac cac acg act aag    1680
Ser Cys Ser Val Val His Glu Gly Leu His Asn His His Thr Thr Lys
        530                 535                 540
agc ttc tcc cgg act ccg ggt aaa tga                                1707
Ser Phe Ser Arg Thr Pro Gly Lys
545                 550
```

[0110]

SEQUENCE ID NO: 14

Length: 568

Type: amino acid

Topology: linear

Type of sequence: peptide

Sequence:

```
Met Pro Leu Leu Leu Leu Leu Leu Leu Leu Pro Ser Pro Leu His Pro
-16 -15                 -10                 -5
His Pro Ile Cys Glu Val Ser Lys Val Ala Ser His Leu Glu Val Asn
 1           5                   10                  15
Cys Asp Lys Arg Asn Leu Thr Ala Leu Pro Pro Asp Leu Pro Lys Asp
             20                  25                  30
Thr Thr Ile Leu His Leu Ser Glu Asn Leu Leu Tyr Thr Phe Ser Leu
            35                  40                  45
Ala Thr Leu Met Pro Tyr Thr Arg Leu Thr Gln Leu Asn Leu Asp Arg
    50                  55                  60
Cys Glu Leu Thr Lys Leu Gln Val Asp Gly Thr Leu Pro Val Leu Gly
65                  70                  75                  80
Thr Leu Asp Leu Ser His Asn Gln Leu Gln Ser Leu Pro Leu Leu Gly
                85                  90                  95
Gln Thr Leu Pro Ala Leu Thr Val Leu Asp Val Ser Phe Asn Arg Leu
            100                 105                 110
Thr Ser Leu Pro Leu Gly Ala Leu Arg Gly Leu Gly Glu Leu Gln Glu
            115                 120                 125
Leu Tyr Leu Lys Gly Asn Glu Leu Lys Thr Leu Pro Pro Gly Leu Leu
            130                 135                 140
Thr Pro Thr Pro Lys Leu Glu Lys Leu Ser Leu Ala Asn Asn Asn Leu
145                 150                 155                 160
Thr Glu Leu Pro Ala Gly Leu Leu Asn Gly Leu Glu Asn Leu Asp Thr
                165                 170                 175
Leu Leu Leu Gln Glu Asn Ser Leu Tyr Thr Ile Pro Lys Gly Phe Phe
                180                 185                 190
```

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```
Gly Ser His Leu Leu Pro Phe Ala Phe Leu His Gly Asn Pro Trp Leu
        195                 200                 205
Cys Asn Cys Glu Ile Leu Tyr Phe Arg Arg Trp Leu Gln Asp Asn Ala
        210                 215                 220
Glu Asn Val Tyr Val Trp Lys Gln Gly Val Asp Val Lys Ala Met Thr
225                 230                 235                 240
Ser Asn Val Ala Ser Val Gln Cys Asp Asn Ser Asp Lys Phe Pro Val
                245                 250                 255
Tyr Lys Tyr Pro Gly Lys Gly Cys Pro Thr Leu Gly Asp Glu Gly Asp
                260                 265                 270
Thr Asp Leu Tyr Asp Tyr Tyr Pro Glu Glu Asp Thr Glu Gly Asp Lys
        275                 280                 285
Val Arg Ala Thr Arg Thr Val Val Lys Phe Pro Thr Lys Ala His Thr
        290                 295                 300
Thr Pro Trp Gly Leu Phe Tyr Ser Trp Ser Thr Ala Ser Leu Asp Glu
305                 310                 315                 320
Pro Arg Gly Pro Thr Ile Lys Pro Cys Pro Pro Cys Lys Cys Pro Ala
                325                 330                 335
Pro Asn Leu Leu Gly Gly Pro Ser Val Phe Ile Phe Pro Pro Lys Ile
                340                 345                 350
Lys Asp Val Leu Met Ile Ser Leu Ser Pro Ile Val Thr Cys Val Val
                355                 360                 365
Val Asp Val Ser Glu Asp Asp Pro Asp Val Gln Ile Ser Trp Phe Val
        370                 375                 380
Asn Asn Val Glu Val His Thr Ala Gln Thr Gln Thr His Arg Glu Asp
385                 390                 395                 400
Tyr Asn Ser Thr Leu Arg Val Val Ser Ala Leu Pro Ile Gln His Gln
                405                 410                 415
Asp Trp Met Ser Gly Lys Glu Phe Lys Cys Lys Val Asn Asn Lys Asp
                420                 425                 430
Leu Pro Ala Pro Ile Glu Arg Thr Ile Ser Lys Pro Lys Gly Ser Val
        435                 440                 445
Arg Ala Pro Gln Val Tyr Val Leu Pro Pro Pro Glu Glu Glu Met Thr
        450                 455                 460
Lys Lys Gln Val Thr Leu Thr Cys Met Val Thr Asp Phe Met Pro Glu
465                 470                 475                 480
Asp Ile Tyr Val Glu Trp Thr Asn Asn Gly Lys Thr Glu Leu Asn Tyr
                485                 490                 495
Lys Asn Thr Glu Pro Val Leu Asp Ser Asp Gly Ser Tyr Phe Met Tyr
                500                 505                 510
Ser Lys Leu Arg Val Glu Lys Lys Asn Trp Val Glu Arg Asn Ser Tyr
        515                 520                 525
Ser Cys Ser Val Val His Glu Gly Leu His Asn His His Thr Thr Lys
```

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```
     530                  535                 540
Ser Phe Ser Arg Thr Pro Gly Lys
545                 550
```

[Brief Explanation of the Drawings]

[Fig. 1] outlines the construction of GPIb-mIgG1Fc expression system.

[Fig. 2] outlines the construction of GPIb-mIgG2aFc expression system.

[Fig. 3] shows binding amount of immobilized von Willebrand factor and a chimeric protein plotted against amount of botrocetin (ELISA).

[Fig. 4] shows activity of inhibition substance for the binding of von Willebrand factor and a chimeric protein (ELISA).

[Fig. 5] shows activity of inhibition substance for the binding of von Willebrand factor and a chimeric protein (Eu-labeling method).

[Fig. 6] shows exemplary quantification of glycocalicin in human plasma (ELISA).

[Fig. 7] shows exemplary quantification of glycocalicin in human plasma (Eu-labeling method).

[Name of docunet] Abstract

[Summary]

[Problem]

A method for conveniently detecting binding between the von Willebrand factor and glycoprotein Ib and a means to be used therein.

[Means to solve the problem]

The von Willebrand factor fixed in a reactor immobilized in a reaction vessel in the presence of bottrocetin is bound to a chimeric protein constructed by fusing the carboxyl terminal of a partial protein containing the von Willebrand factor-binding site of glycoprotein Ib with the amino terminal of the Fc region of an immunoglobulin molecule. Then the Fc region of the above immunoglobulin molecule is detected to thereby detect the binding between the von Willebrand factor and the glycoprotein Ib or inhibition of this binding.

[Selected drawing] Fig. 4